The Journal
of
Biological Chemistry

Volume 13

1912-1913

Reprinted by arrangement with the American Society of Biological Chemists, Inc

JOHNSON REPRINT CORPORATION
New York, New York

THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A HERTER MEMORIAL FUND

VOLUME XIII

BALTIMORE

1912–13

THE JOURNAL OF BIOLOGICAL CHEMISTRY

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striking difference between the salts of amino-acids on the one hand, and the salts of peptides and peptones on the other As stated in the previous paper, "the copper salts of the amino-acids in alkaline solutions, (contrary to statements in scientific literature,) particularly on warming or on boiling, precipitate copper as the hydroxide, quantitatively Peptides and peptones, on the other hand, give very little or no hydroxide under the same conditions "

The explanation of this important difference may possibly be due to one or more of the following reasons (1) A marked difference in the constitution of these salts, (2) A change of constitution brought about by the excess alkali, (3) A change in the form or condition of the copper due to the presence of the protein-like substances, *e g*, "colloidal copper "[5]

The fact that peptides do not form copper salts strictly in accordance with the number of free carboxyl groups,[6] as do most of the amino-acids, favors the theory of a difference in the constitution as the cause of their abnormal behavior on treatment with excess alkali The fact that most copper salts of peptides and peptones change color on treatment with alkali, giving the so-called "biuret color," indicates that a change of structure in these peptides takes place on adding excess alkali The fact that a similar phenomenon described by Paal and Leuze[7] was explained as a "colloidal pro cess" gives a certain degree of plausibility to the third reason As will be seen, the data given in the latter part of this paper support the first two explanations, while not many facts support the third [8]

As Leuchs,[9] conjunctively with Manasse and La Forge, has considerable experimental basis for considering that carbethoxylglycyl-glycine ester and allied compounds have two forms, the lactim and the lactam, we believed at first that the lactim form was the chief cause for the copper not precipitating in alkaline solutions, behaving in this respect like other hydroxy-acids, such

[5] Paal and Leuze *Ber d deutsch chem Gesellsch*, xxxix, p 1545

[6] Fischer *Untersuchungen über Amino-säuren, Polypeptide und Proteine* 1906, p 50

[7] *Loc cit*

[8] Copper salts in weak alkaline solutions dialyze, but when the alkali is strong, they combine with the membrane and cause difficulties

[9] *Ber d deutsch chem Gesellsch*, xl, p 3235, xli, p 2586

THE COPPER COMPLEXES OF AMINO-ACIDS, PEPTIDES AND PEPTONES[1]

FIRST PAPER

By P. A. KOBER AND K. SUGIURA

(From the Harriman Research Laboratory, Roosevelt Hospital, New York City.)

(Received for publication, August 8, 1912.)

INTRODUCTION

That proteins and their constituents combine with heavy metals to form "complexes" has been known for some time. Among these copper has received the most attention and considerable work has been done on the copper complexes of amino-acids: they have been isolated,[2] analyzed and studied by chemical and physical methods. On the other hand the complexes of peptides and peptones have received only a superficial and qualitative study. This is not due to the unimportance of peptide copper salts, for the behavior of peptide and peptone copper salts on treatment with an excess of alkali has been and is yet the most reliable test known for protein-like substances. The real reason for this lack of investigation is, without doubt, due first to the difficulty of obtaining peptides and peptones pure, and second to the fact that the copper salts of these substances are not easily crystallized. It is our belief that a quantitative study of these copper salts[3] will throw some light on the constitution of proteins and it is therefore our intention to continue from time to time our efforts in this field.

In a previous preliminary communication[4] one of us showed a

[1] Read before the Section on Organic Chemistry, International Congress of Applied Chemistry, New York, Sept., 1912.

[2] Ley: *Zeitschr. f. Electrochem.*, xi, p. 954; Schiff: *Ann. d. Chem.*, xliii, p. 123; Bruni: *Chem. Zentralblatt*, p. 824, 1904; Ley and Krafft: *Ber. d. deutsch. chem. Gesellsch.*, xl, p. 697, 1907; Fischer: *Untersuchungen über Amino-säuren, Polypeptide und Proteine*, 1906.

[3] The term *copper salt*, used here and throughout the paper, has reference to a true salt, *i. e.*, the product of the action of amino-acids and protein-like substances in general on cupric hydroxide, oxide or carbonate.

[4] Kober: this *Journal*, x, p. 9, 1911.

striking difference between the salts of amino-acids on the one hand, and the salts of peptides and peptones on the other As stated in the previous paper, "the copper salts of the amino-acids in alkaline solutions, (contrary to statements in scientific literature,) particularly on warming or on boiling, precipitate copper as the hydroxide, quantitatively Peptides and peptones, on the other hand, give very little or no hydroxide under the same conditions"

The explanation of this important difference may possibly be due to one or more of the following reasons (1) A marked difference in the constitution of these salts, (2) A change of constitution brought about by the excess alkali, (3) A change in the form or condition of the copper due to the presence of the protein-like substances, *e g*, "colloidal copper"[5]

The fact that peptides do not form copper salts strictly in accordance with the number of free carboxyl groups,[6] as do most of the amino-acids, favors the theory of a difference in the constitution as the cause of their abnormal behavior on treatment with excess alkali The fact that most copper salts of peptides and peptones change color on treatment with alkali, giving the so-called "biuret color," indicates that a change of structure in these peptides takes place on adding excess alkali The fact that a similar phenomenon described by Paal and Leuze[7] was explained as a "colloidal process" gives a certain degree of plausibility to the third reason As will be seen, the data given in the latter part of this paper support the first two explanations, while not many facts support the third [8]

As Leuchs,[9] conjunctively with Manasse and La Forge, has considerable experimental basis for considering that carbethoxyl-glycyl-glycine ester and allied compounds have two forms, the lactim and the lactam, we believed at first that the lactim form was the chief cause for the copper not precipitating in alkaline solutions, behaving in this respect like other hydroxy-acids, such

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[9] *Ber d deutsch chem Gesellsch*, xl, p 3235, xli, p 2586

as lactic, tartaric, etc , but owing to the lack of spectrographic data we cannot say whether this really plays a part or not

As part of our results are at variance with some obtained previously by other investigators, our technique and results will be described and discussed before data are weighed in support of any theory

TECHNIQUE

Fischer,[10] Abderhalden,[11] and others prepared the copper salts of amino-acids, peptides and peptones by boiling with cupric oxide We found that they were formed best at a low temperature, with cupric hydroxide, preferably in an ice mixture Since some heat of neutralization makes the process an exothermal one, it is not unexpected that the reaction will be complete at a low temperature A further objection to boiling lies in the fact that a few of these salts have a slight tendency to hydrolyze Where a reaction can be brought about at a freezing temperature the danger of decomposing unstable peptides is obviously reduced to a minimum

The form of the copper hydroxide is very important as on standing it is dehydrated with the formation of cupric oxide That cupric oxide is slower in its reaction with these peptides can readily be seen from the constitution of the copper salts [12]

The best conditions for forming these copper salts quantitatively are as follows

a For soluble substances

For every 0 1 gram of amino-acid or peptide 5 cc of 5 per cent cupric chloride solution are diluted to 150 or 200 cc with ice-cold distilled water and neutralized with alkali This can be done most suitably by mixing 100 grams of fine ice with 5 cc of 5 per cent cupric chloride solution and 100 cc of distilled water and neutralizing with $\frac{N}{5}$ alkali, using phenolphthalein as an indicator The neutralized mixture is then filtered, the ice being placed on the filter also in order to keep the precipitated cupric hydroxide cool The hydroxide is washed once or twice with cold distilled water on the filter paper, removed with the ice to the cooled 10–20 cc solution of the amino-acid or peptide and stirred for from five to ten minutes

On filtering off the excess cupric hydroxide and washing thoroughly, the solution will contain the copper salts of all the substances As a rule, the solutions require boiling, to decompose the very appreciable amount

[10] Fischer *loc cit*

[11] Abderhalden *Ber d deutsch chem Gesellsch* , xxxx, p 2746

[12] *Amer Chem Journ* , Nov , 1912

of carbamino salts that are formed with CO_2 of the air, and this may be done before filtering To ascertain the amount of copper hydroxide dissolved, the copper salt may be titrated directly (with 0 04 N $Na_2S_2O_3$) by adding 5–10 cc of glacial acetic acid and 2–4 grams KI The amount of carbamino salts formed is increased by the presence of sodium chloride whereas alcohol, on the contrary, hinders their formation

b For insoluble substances with slightly soluble copper salts

If the amino-acid or peptide is insoluble, 10–20 cc of $\frac{N}{10}$ ammonia are added to the sample and it is stirred until dissolved If necessary the mixture may be heated to hasten solution After cooling the solution in an ice mixture, cold cupric hydroxide, made as above, is added and stirred for from five to ten minutes, 150 cc of water are then added and the solution filtered after thorough shaking The precipitate is washed well with hot water and the filtrate and wash waters are concentrated The amount of copper hydroxide dissolved may be titrated iodimetrically in the usual way Controls on this method, using copper hydroxide and 20 cc of $\frac{N}{10}$ ammonia, gave only 0 0003 to 0 0005 gram of copper oxide in the filtrate

c For insoluble substances with quite insoluble copper salts

Where the copper salt crystallizes out and is filtered off with the excess copper hydroxide, it is necessary to separate the two precipitates

Very satisfactory reagents for this purpose are the bicarbonates of sodium and potassium Using 10 and 20 per cent solutions of $KHCO_3$ we have obtained the results given below on leucine, tryptophane, cystine, amino-*n*-caproic acid and phenylglycine

The amino substance is treated as in *b*, but the excess copper hydroxide, mixed with the insoluble copper salt, is then treated with 20 cc of 20 per cent $KHCO_3$ and washed with small lots of 10 per cent $KHCO_3$ until the filtrate shows only traces of copper To determine directly the amount of copper hydroxide dissolved, the final residue of copper salt is then transferred with the filter paper to the first filtrate and after being dissolved in dilute hydrochloric[13] acid is titrated as before

On page 13 under experimental notes we give a few experiments on the solubility of these "insoluble" copper salts in various concentrations of potassium bicarbonate In the near future we hope to give the details of more experiments along these lines, especially on the determination of amino-acids in the presence of polypeptides

Boiling directly with copper hydroxide or oxide will result without doubt in the incomplete formation of copper salts, this fact helps to explain the unexpected results obtained by Abderhalden and Hirsch on *d*-alanyl-*l*-leucyl-isoleucine and its glycyl derivatives (see page 7)

[13] As large amounts of potassium acetate retard the iodimetric titrations of copper, we neutralize with pure HCl

SUMMARY OF DATA

Amino-acid copper salts

The results with our technique on α amino-acids confirm the analyses made by previous investigators of isolated copper salts, which have without exception the general formula CuA_2, where A represents one molecule of monobasic α-amino-acid

A few examples will suffice

Monobasic amino-acids

Substances	Method of preparation	Weight of sample	CuO in filtrate after adding 3 cc N NaOH (CO_2 free) and boiling	CuO in precipitate after adding 3 cc N NaOH (CO_2 free) and boiling	Theoretical weight CuO calculated for this sample	Total CuO as percentage of theoretical
		grams	*grams*	*grams*	*grams*	*per cent*
Glycine	*a*	0 1015	0 0000	0 0536	0 0538	99 6
Alanine	*a*	0 1019	0 0000	0 0453	0 0455	99 6
Aminobutyric acid	*b*	0 1022	0 0004	0 0403	0 0395	102 0
Active valine	*a*	0 0944	0 0015	0 0301	0 0321	93 8
Leucine	*c*	0 1010	0 0054	0 0361	0 0307	102 6
Normal aminocaproic acid	*c*	0 1003	0 0002	0 0315	0 0305	105 7
Isoleucine	*a*	0 1014	0 0011	0 0307	0 0308	103 2
Active proline	*a*	0 1018	0 0078	0 0269	0 0352	98 6
Phenylalanine	*b*	0 1001	0 0000	0 0244	0 0242	100 8
Phenylglycine	*c*	0 1011	0 0000	0 0280	0 0266	105 3
Tyrosine	*b*	0 1027	0 0007	0 0263	0 0226	116 4
Tryptophane*	*b*	0 1022	0 0000	0 0183	0 0199	92 0
Asparagine	*b*	0 1005	0 0005	0 0308	0 0303	101 7
Sarcosine hydrochloride†	*a*	0 1002	0 0021	0 0299	0 0315	101 6
Lysine picrate‡	*a*	0 1006	0 0006	0 0097	0 0107	96 3
Arginine dinitrate	*a*	0 1010	0 0011	0 0121	0 0134	98 5
Histidine dihydrochloride§	*a*	0 1000	0 0161	0 0005	0 0175	94 9

*If more than 0 1 gram tryptophane is used method *c* must be added to this technique

†All acid salts such as hydrochlorides nitrates, etc are neutralized with $\frac{N}{10}$ alkali, using phenolphthalein as an indicator before treating with copper hydroxide as in method *a*

‡Our thanks are due to Dr Levene of the Rockefeller Institute for furnishing us with isoleucine arginine and lysine

§Histidine forms a complex salt as do the other monobasic amino-acids and on treatment with excess alkali changes its color but little Only on boiling the color changes towards a biuret It is not a clear color but smoky and makes the solution look very dark Characteristic is the deep red color to which the alkaline solution turns on the addition of acid We expect to make this a basis for the colorimetric qualitative and quantitative estimation of histidine

With other amino-acids, whose NH_2 group is in the β-position, Fischer found the formula to be CuA_2, where A is monobasic amino-acid, except (as in the case of isoserine) when an oxy group is in the α-position, where the formula is CuA

We have obtained the same results with isoserine,[14] using the technique *a*

Substance	Weight of sample	CuO in filtrate after adding 3 cc N NaOH (CO_2 free) and boiling	CuO in precipitate after adding 3 cc N NaOH (CO_2 free) and boiling	Total CuO	Theoretical weight CuO calculated for this sample	Total CuO as percentage of theoretical
	grams	*grams*	*grams*	*grams*	*grams*	*per cent*
Isoserine	0 1010			0 0807	0 0765	105 5
Isoserine	0 1009	0 0299	0 0499	0 0798	0 0764	104 5

According to Fischer,[15] when the amino group is in the γ-, δ-, or ϵ-position (regardless of an hydroxy group in the α position) no copper salts are formed

Our results confirm the previous figures on dibasic salts having a general formula CuA, where A is a dibasic acid, such as aspartic, glutaminic, cystinic, etc

Dibasic amino-acids

Substance	Method of preparation	Weight of sample	CuO in filtrate after adding 3 cc N NaOH (CO_2 free) and boiling	CuO in precipitate after adding 3 cc N NaOH (CO_2 free) and boiling	Theoretical weight CuO calculated for this sample	Total CuO as percentage of theoretical
		grams	*grams*	*grams*	*grams*	*per cent*
Cystine	*c*	0 1008	0 0000	0 0331	0 0334	99 1
Aspartic acid*	*a*	0 0997	0 0009	0 0579	0 0596	98 6
Glutaminic acid*	*a*	0 1025	0 0004	0 0518	0 0554	94 9

*Before boiling the solutions of copper salts are diluted to 150 cc with water

[14] Made according to Fischer's directions from epichlorhydrin *Ber d deutsch chem Gesellsch*, xxxv, p 3787

[15] E Fischer and L Zemplen *Ber d deutsch chem Gesellsch*, xlii, p 4883, 1909

Polypeptide copper salts

Out of a hundred or more polypeptides, Fischer, Abderhalden and their collaborators have analyzed only about eight of the polypeptide copper salts, and these, with three exceptions (nos VI, VII and VIII) were insoluble and crystallized out In such cases there can be no doubt as to the purity of the product and the results are confirmed by our work on the more soluble copper peptides These cases are as follows

I (Leucyl-glycine copper)$_2$O + H$_2$O[16] or $(C_8H_{16}O_3N_2Cu)_2O + H_2O$

Fischer concluded that one molecule of copper hydroxide combined with one molecule of leucyl-glycine and that two molecules of the copper-leucyl-glycine are connected by an oxygen atom Although we have found one molecule of copper hydroxide to one of leucyl-glycine, we do not believe that an oxygen atom connects two molecules of copper-leucyl-glycine We have determined the molecular weight of this salt by the cryoscopic method, and find it to have a molecular weight consistent with the formula leucyl-glycine-Cu,

II (Phenyl-glycine-glycine)$_1$copper,[17] $C_{10}H_{10}N_2O_3Cu$

III (*l*-Leucyl-*l*-histidine)$_1$copper,[18] $C_{12}H_{18}O_3N_4Cu$

IV (Alanyl-*l*-tryptophane)$_1$copper,[19] $C_{14}H_{15}N_3O_3Cu$

V (*l*-Prolyl-*l*-phenylalanine)$_1$copper,[20] $C_{14}H_{16}N_2O_3Cu + 3\frac{1}{2}H_2O$

VI (*d*-Alanyl-*l*-leucyl-isoleucine)$_2$copper,[21] $(C_{15}H_{27}O_4N_3)_2Cu$

VII (Glycyl-*d*-alanyl-*l*-leucyl-isoleucine)$_2$copper,[22] (peptide)$_2$Cu

VIII (Tri-glycyl-glycine)$_2$copper,[23] (tetrapeptide)$_2$Cu

Out of the large number of peptide copper salts examined by Fischer, Abderhalden and ourselves, the last three form an apparent exception to the rule There are good reasons to believe that

[16] Fischer *Ann d Chem*, cccxl, p 145

[17] Fischer *ibid*, p 195

[18] Fischer and L H Cone *ibid*, ccclxiii, p 107

[19] Abderhalden and M Kempe *Ber d deutsch chem Gesellsch*, xl, p 2737

[20] Fischer *ibid*, xlii, p 4752

[21] Abderhalden and Hirsch *ibid*, xliii, p 2439

[22] *Ibid*

[23] Curtius *ibid*, xxxvii, p 1294

the formulas given for VI, VII and VIII do not represent true copper salts, as the methods used in each case are no guarantee of the complete formation of the salts

Using the technique described above, we have formed the copper salts of the following peptides, and have found the results to be consistent with the formula, (peptide)$_1$Cu$_1$

Dipeptides

SUBSTANCE	WEIGHT OF SAMPLE	CuO IN FILTRATE AFTER ADDING 3 CC N NaOH (CO₂ FREE) AND BOILING	CuO IN PRECIPITATE AFTER ADDING 3 CC N NaOH (CO₂ FREE) AND BOILING	TOTAL CuO	THEORETICAL WEIGHT CuO CALCULATED FOR THIS SAMPLE	TOTAL CuO AS PERCENTAGE OF THEORETICAL
	grams	*grams*	*grams*	*grams*	*grams*	*per cent*
Glycyl-glycine	0 1123	0 0536	0 0070	0 0606	0 0616	98 4
Glycyl-alanine	0 1006	0 0501	0 0049	0 0550	0 0548	100 4
Glycyl-*d*-alanine	0 1011	0 0524	0 0006	0 0530	0 0550	96 5
Glycyl-aminobutyric acid	0 1007	0 0437	0 0030	0 0467	0 0500	93 4
Glycyl-valine	0 1012	0 0400	0 0062	0 0462	0 0462	100 0
Glycyl-*d*-valine	0 1015	0 0390	0 0059	0 0449	0 0464	96 8
Glycyl-leucine	0 1005	0 0380	0 0022	0 0402	0 0425	94 6
Glycyl-*l*-leucine	0 1006	0 0370	0 0036	0 0406	0 0425	95 5
Glycyl-amino-*n*-caproic acid	0 0963	0 0377	0 0014	0 0391	0 0407	96 1
Glycyl-asparagine	0 1028	0 0397	0 0031	0 0428	0 0433	98 8
Glycyl-phenylglycine	0 1021	0 0355	0 0030	0 0385	0 0390	98 7
Glycyl-*d*-phenylglycine	0 1022	0 0358	0 0010	0 0368	0 0391	94 1
Glycyl-tyrosine	0 0545	0 0142	0 0043	0 0185	0 0181	102 2
Glycyl-tryptophane	0 1016	0 0257	0 0010	0 0267	0 0310	86 1
Alanyl-glycine	0 1007	0 0516	0 0032	0 0548	0 0548	100 0
l-Alanyl-glycine	0 1005	0 0496	0 0054	0 0550	0 0547	100 5
d-Alanyl-*d*-alanine	0 1001	0 0465	0 0041	0 0506	0 0497	101 8
Aminobuteryl-glycine	0 1010	0 0467	0 0045	0 0512	0 0502	102 0
Valyl-glycine	0 1005	0 0410	0 0059	0 0469	0 0459	102 2
Leucyl-glycine	0 0901	0 0344	0 0041	0 0385	0 0381	101 0
Leucyl-leucine	0 1006	0 0288	0 0014	0 0302	0 0328	92 1
l-Leucyl-*d*-leucine	0 1004	0 0266	0 0021	0 0287	0 0327	87 8
d-Leucyl-*l*-leucine	0 1011	0 0268	0 0003	0 0271	0 0330	82 1
d-Leucyl-*d*-leucine	0 1007	0 0294	0 0013	0 3007	0 0328	93 6
Amino-*n*-caproic-glycine	0 0664	0 0257	0 0015	0 0272	0 0281	96 8
Leucyl-asparagine	0 1004	0 0205	0 0010	0 0215	0 0326	66 0

Tripeptides [1]

Substance	Weight of sample	CuO in filtrate after adding 3 cc 1/N NaOH (CO$_2$ free) and boiling	CuO in precipitate after adding 3 cc N NaOH (CO$_2$ free) and boiling	Total CuO	Theoretical weight CuO calculated for this sample	CuO in filtrate as percentage of theoretical	Total CuO as percentage of theoretical
	grams	*grams*	*grams*	*grams*	*grams*	*per cent*	*per cent*
Glycyl-glycyl-glycine	0 1003	0 0370	0 0006	0 0376	0 0422	87 7	89 1
Glycyl-glycyl-alanine	0 1021	0 0356	0 0064	0 0420	0 0400	89 0	105 0
Glycyl-glycyl-aminobutyric acid	0 1011	0 0382	0 0006	0 0388	0 0371	103 0	104 6
Glycyl-glycyl-valine	0 0516	0 0155	0 0005	0 0160	0 0178	87 1	89 9
Glycyl-glycyl-leucine	0 1010	0 0279	0 0044	0 0323	0 0328	86 4	98 5
Glycyl-glycyl-*l*-leucine	0 1020	0 0287	0 0024	0 0311	0 0331	86 7	94 0
Glycyl-*d*-alanyl-*d*-alanine	0 0536	0 0144	0 0001	0 0145	0 0192	75 0	75 5
Glycyl-leucyl-glycine	0 1013	0 0293	0 0004	0 0297	0 0329	88 5	90 3
Alanyl-glycyl-glycine	0 0987	0 0254	0 0031	0 0285	0 0387	65 6	73 6
d Alanyl-glycyl-glycine	0 0492	0 0168	0 0014	0 0182	0 0193	87 0	94 3
Alanyl-leucyl-glycine	0 1012	0 0274	0 0037	0 0311	0 0311	88 1	100 0
Amino-buteryl-glycyl-glycine	0 1011	0 0350	0 0028	0 0378	0 0371	94 3	101 9
Valyl-glycyl-glycine	0 1023	0 0288	0 0027	0 0315	0 0352	81 8	89 5
Leucyl-glycyl-glycine	0 1013	0 0283	0 0057	0 0340	0 0329	86 1	103 3
l-Leucyl-glycyl-glycine	0 1011	0 0284	0 0043	0 0327	0 0328	86 6	99 7
l-Leucyl-glycyl-*d*-alanine	0 0569	0 0161	0 0008	0 0169	0 0175	92 0	96 6
Leucyl-alanyl-glycine	0 1002	0 0289	0 0040	0 0329	0 0308	93 8	106 8
Leucyl-alanyl-alanine	0 1017	0 0272	0 0050	0 0322	0 0296	91 9	108 8
l-Leucyl-*d*-alanyl-*d*-alanine	0 1014	0 0262	0 0033	0 0295	0 0295	88 8	100 0
Amino-*n*-caproyl-glycyl-glycine	0 1003	0 0304	0 0035	0 0339	0 0325	93 5	104 3

[1] The majority of the peptides used in this study were prepared or collected by the late Dr Arthur H Koelker A small number (four dipeptides and three tripeptides) were made according to Fischer's directions, by Dr H Hager and ourselves, in this laboratory Glycyl-tryptophane was obtained in very pure crystalline form through the kindness of Kalle and Co

Tetrapeptides

SUBSTANCE	WEIGHT OF SAMPLE	CuO IN FILTRATE AFTER ADDING 3 CC N NaOH (CO_2 FREE) AND BOILING	CuO IN PRECIPITATE AFTER ADDING 3 CC N NaOH (CO_2 FREE) AND BOILING	TOTAL CuO	THEORETICAL WEIGHT CuO CALCULATED FOR THIS SAMPLE	CuO IN FILTRATE AS PERCENTAGE OF THEORETICAL	TOTAL CuO AS PERCENTAGE OF THEORETICAL
	grams	*grams*	*grams*	*grams*	*grams*	*per cent*	*per cent*
Alanyl-diglycyl-glycine	0 1008	0 0267	0 0017	0 0284	0 0308	86 7	92 2
Aminobuteryl-diglycyl-glycine	0 1050	0 0260	0 0037	0 0297	0 0305	85 2	94 1
Leucyl-diglycyl-glycine*	0 1008	0 0239	0 0030	0 0269	0 0265	90 2	101 5
n-Amino-caproyl-diglycyl-glycine	0 1017	0 0202	0 0005	0 0207	0 0268	75 4	77 2

* Filtered without boiling to decompose carbamino salts

Using our same technique it was of interest to see the amount of copper hydroxide dissolved by peptones

Peptones, etc

SUBSTANCE	WEIGHT OF SAMPLE	CuO IN FILTRATE AFTER ADDING 3 CC N NaOH (CO_2 FREE) AND BOILING	CuO IN PRECIPITATE AFTER ADDING 3 CC N NaOH (CO_2 FREE) AND BOILING	MOLECULAR WEIGHT* CALCULATED FROM TOTAL CuO
	grams	*grams*	*grams*	
"Roche" peptone	0 1110	0 0302	0 0173	186
"Ereptone"	0 1005	0 0064	0 0133	406
"Merck" peptone	0 1020	0 0044	0 0002	1765
"Witte's" peptone	0 1025	0 0041	0 0000	1990
"Witte's" peptone	0 1046	0 0042	0 0000	1982

*This calculation is based on the assumption that one molecule of peptone as in the case of the peptides combined with only one molecule of copper hydroxide

The results on the peptides show unmistakably that one molecule of peptide, whatever number of amino-acids it may contain, combines with only one molecule of copper hydroxide This interest-

ing fact will no doubt give us an easy method of determining the molecular weights of peptides, provided the copper salts can be separated from the excess copper hydroxide

The questions that remain to be solved, namely, how are these copper salts formed and what is their structure, will be taken up in a separate paper,[25] in conjunction with the biuret configuration

SUMMARY

1 We have developed a technique for making, quantitatively, copper salts of (a) soluble amino acids and peptides, (b) insoluble amino-acids having soluble copper salts, (c) insoluble amino-acids having insoluble copper salts

2 We have formed copper salts of twenty-six dipeptides, twenty tripeptides and four tetrapeptides, quantitatively, in solution, and have found the results to be consistent with the formula, (peptide)$_2$Cu This supports the work of Fischer and Abderhalden on five isolated salts

3 We have found (*a*) That on an average 99 per cent of the copper of all amino-acid salts (except that of histidine) is precipitated as oxide when treated with a certain excess of alkali [26]

(*b*) That on an average 6 4 per cent of the copper of dipeptide salts is precipitated as oxide with the same excess of alkali

(c) That on an average 6 3 per cent of the copper of tripeptide[27] salts is precipitated as oxide with the same excess of alkali

(*d*) That on an average 7 3 per cent of the copper of tetrapeptide salts is precipitated as oxide with the same excess of alkali

Our thanks are due to Dr Wm G Lyle for his encouragement in this work, and for placing at our disposal the excellent collection of peptides and amino-acids belonging to the late Dr A H Koelker, and to Miss Calm M Hoke for much assistance in preparing this article for publication

[25] *Amer Chem Journ*, Nov, 1912

[26] Using 3-5 cc N CO_2-free NaOH for every 0 1 gram substance and boiling

[27] Assuming all the substances to be perfectly pure, which of course they are not It is probable that perfectly pure tri- and tetra-peptides will not precipitate any of the copper of their salts under the conditions given above We are convinced that impurities will account for most of the precipitation

EXPERIMENTAL NOTES

a Copper oxide as a reagent

In our first work on copper salts we used black copper oxide obtained in the open market as a reagent, but found that with peptides it reacted too slowly Then we tried the commercial basic carbonate, and found it more useful, but not quite satisfactory *Freshly prepared copper hydroxide, however, was found not only efficient but almost instantaneous in its reaction at 0°C* The amount of surface is, of course, a factor in the reaction, and therefore a gelatinous precipitate is, other things being equal, preferable to a coarse granular precipitate

As Fischer and Abderhalden used suspended copper oxide in making their salts, it was necessary to investigate the relative efficiency of this reagent on various amino-acids and peptides *when boiled*

The following table gives the results with *freshly made copper hydroxide* and *freshly made copper oxide suspensions on boiling* The latter were made by allowing a suspension of the hydroxide to stand several days in the laboratory, during which time it changed its color from blue to almost black

SUBSTANCE	WEIGHT OF SAMPLE	CuO DISSOLVED AFTER BOILING 10 MINUTES WITH Cu (OH)₂	CuO DISSOLVED AFTER BOILING 10 MINUTES WITH CuO	THEORETICAL WEIGHT OF CuO	CuO AS FOUND TO THEORETICAL
	grams	*grams*	*grams*	*grams*	*per cent*
Glycine	0 1011	0 0564		0 0536	105 2
Glycine	0 1016		0 0558	0 0539	103 5
Alanine	0 1034	0 0482		0 0462	104 3
Alanine	0 1021		0 0472	0 0456	103 5
Alanyl-glycine	0 1013	0 0562		0 0551	103 0
Alanyl-glycine	0 1001		0 0515	0 0547	94 5
Leucyl-glycine	0 1023	0 0435		0 0433	100 5
Leucyl-glycine	0 1015		0 0435	0 0429	101 4
Alanyl-leucyl-glycine	0 1004	0 0210		0 0308	68 2
Alanyl-leucyl-glycine	0 1010		0 0137	0 0310	44 2
Glycyl-leucyl-glycine	0 1011	0 0242		0 0328	73 8
Glycyl-leucyl-glycine	0 1003		0 0150	0 0325	46 2
Alanyl-di-glycyl-glycine	0 1003		0 0195	0 0307	63 2

These results show that boiling, while suitable for amino-acids and dipeptides, cannot be used with tri- and tetra-peptides, and will easily account for the abnormal results obtained by Abderhalden and Hirsch [28]

b Bicarbonates as solvents for copper hydroxide

When cystine, tryptophane, leucine and other amino-acids, whose copper salts are insoluble, are treated as in method *c* (see p 4), the copper salts are found almost wholly [29] in the precipitate, mixed with the excess copper hydroxide

In order to show the effect of different concentrations of the bicarbonate on the solubility of copper hydroxide and the "insoluble" copper salts, the following preliminary experiments were made

Pure copper salts of the amino-acids were made as described under *c* and a small portion (sufficient to leave a slight excess undissolved) was stirred constantly for five minutes in 25 cc of 5 per cent, 10 per cent and 20 per cent potassium bicarbonate, controls were made on 25 cc of distilled water [30]

SUBSTANCE	CuO DISSOLVED IN 25 CC H_2O	CuO DISSOLVED IN 25 CC 5 PER CENT $KHCO_3$	CuO DISSOLVED IN 25 CC 10 PER CENT $KHCO_3$	CuO DISSOLVED IN 25 CC 10 PER CENT $NaHCO_3$	CuO DISSOLVED IN 25 CC 20 PER CENT $KHCO_3$
	grams	*grams*	*grams*	*grams*	*grams*
Copper hydroxide	0 00000	0 0283	0 0580	0 0565	0 1680
Leucine, copper	0 00020	0 0006	0 0008		0 0015
n-Aminocaproic acid, copper	0 00003	0 0007	0 0016	0 0014	0 0060
Phenylglycine, copper	0 00003	0 0006	0 0012	0 0013	0 0056
Tryptophane, copper	0 00020	0 0004	0 0004	0 0005	0 0007
Cystine, copper	0 00000	0 0000	0 0000	0 0000	0 0001

[28] *Loc cit*

[29] When less than 0 1 gram of tryptophane is used, the copper salt remains in a supersaturated condition in the filtrate and later crystallizes out

[30] Only one concentration of sodium bicarbonate was used, the potassium bicarbonate, on account of its greater solubility, is preferable

A STUDY OF THE MECHANISM OF PHLORHIZIN DIABETES

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(Received for publication, August 7, 1912)

The distinctive feature of phlorhizin diabetes in contrast with other types is the significant diminution in the blood sugar content The mechanism by which this condition of hypoglycaemia is established has been explained by a variety of theories, none of which, however, has received universal acceptance Of the numerous views[1] that have been promulgated two have received considerable attention It was the opinion of v Mering[2] that the drug creates an increased permeability of the kidney for sugar, thus leading to the passage of blood sugar into the urine To meet this drain upon the sugar of the blood an augmented activity of the blood sugar regulating mechanism is supposed to be set up whereby new sugar is formed in the body from some antecedent substance, presumably protein If this explanation is correct one might fairly assume that extirpation or ligation of the kidneys in phlorhizin diabetes by preventing a renal loss of sugar would result in a restoration of sugar to its normal percentage in the blood Minkowski's[3] experiments tend to support this position although, as Levene[4] pointed out, Minkowski obtained an increase of sugar in the blood beyond the normal after extirpation of the kidneys, which theoretically should not result if phlorhizin merely has a specific influence upon the kidney and does not act upon some other mechanism

[1] For a discussion of the problem, cf Macleod *Recent Advances in Physiology and Bio-Chemistry*, Edited by Leonard Hill, 1906

[2] v Mering *Verhandl d 5te Congresses f inn Med* , 1886, p 185

[3] Minkowski *Arch f exp Path u Pharm* , xxxi, p 85, 1893

[4] Levene *Journ of Physiol* , xvii, p 259, 1894–5

A second view of the nature of phlorhizin diabetes, which was advocated by Pavy and others,[5] differs from the first primarily in ascribing to the kidney the power of *producing* sugar. Under the influence of phlorhizin the cells of the renal tubules are supposed to exert a catabolizing action upon something reaching them from the blood, resulting in the liberation of dextrose in a manner comparable to that by which lactose is set free by the cells of the mammary gland.

That the conditions existing in pancreatic and phlorhizin diabetes are similar in many respects is well recognized. Thus in the starving dog the D : N ratios obtained in the two experimental states are somewhat alike. Moreover, it is generally conceded that in phlorhizin diabetes as well as in the condition induced by removal of the pancreas the ability of the organism to utilize dextrose may be somewhat diminished. The distinguishing difference between these two abnormal states is found in the lowered blood sugar content in phlorhizin diabetes and the existence of hyperglycaemia in pancreatic diabetes.

Although numerous investigations have been carried through concerning the relation of ligation or ablation of the kidneys to phlorhizin diabetes a review of the literature fails to reveal an experiment of this nature with animals in *total* diabetes, that is, with a D : N ratio of approximately 3.65. In the work of Minkowski comparison has been made of the blood sugar content of dogs both in phlorhizin and pancreatic diabetes after kidney removal. He found that in pancreatic diabetes hyperglycaemia was in evidence whereas the blood sugar content rose little, if at all, above the normal in phlorhizin diabetes. It appears to the writer, however, that the conditions existing in the two experimental states were too dissimilar for strict comparison. In pancreatic diabetes a *constant* influence is at work for one may reasonably assume that extirpation of the pancreas either initiates a stimulus for, or removes an inhibition from, the sugar regulating mechanism. In phlorhizin diabetes, on the other hand, to produce a similar constant response on the part of the body the animal organism must be continually supplied with the necessary quantity of the drug. Expressed in other words, previous experiments have been per-

[5] Pavy, Brodie and Siau. *Journ. of Physiol.*, xxix, p. 467, 1903.

formed with animals in unlike states, in pancreatic diabetes, a constant stimulus has been present, while little or no attempt has been made to imitate comparable conditions in phlorhizin diabetes The production and maintenance of a D N ratio of 3 65 would appear to satisfy the condition of a constant stimulus Ablation or ligation of the kidneys *under these circumstances* would perhaps constitute an experiment with conditions more strictly comparable with those obtaining in pancreatic diabetes than is true for many of the previous investigations Hitherto, for the most part, little or no attention has been devoted to the question of phlorhizin dosage or to the period of time which phlorhizin might reasonably be expected to exert an influence upon the percentage of sugar in the blood At present it is well recognized that the glycosuric influence of a single injection of phlorhizin persists, in dogs at least, for a few hours only Yet there are records of experiments of the type under discussion demonstrating the possibility that sufficient time had elapsed since the last phlorhizin injection for restoration of blood sugar content by utilization of an excess in the blood, provided a high percentage of sugar in the blood had been temporarily established

The object of the present investigation has been a study of the changes in blood sugar content of animals in phlorhizin diabetes after ligation of the kidneys or suppression of the renal secretory function Two types of experiments were planned In the one, dogs have been brought into a condition of *total* diabetes with phlorhizin The kidneys were then ligatured twice, one ligature being placed around the ureters and the blood vessels, another designed to include any collateral vascular branches The experiments were so planned that the operation was performed upon the kidneys shortly after the morning administration of phlorhizin, and a second injection of the drug was given as usual eight hours subsequent to the first Blood was analyzed for total solids and dextrose content just previous to the operation and at intervals subsequent to ligature of the renal organs

In the other type of experiment the function of the kidney as an excretory organ was practically abolished in fasting phlorhizinized rabbits by the subcutaneous administration of sodium tartrate[6]

[6] Cf Underhill this *Journal,* xii, p 115, 1912

In this instance the blood supply to the kidneys was presumably uninterrupted whereas the escape of sugar from the blood was prevented by the changed character of the tubular epithelium The establishment of such a condition has been found to occur after tartrate injections and has been recorded in a previous paper Blood analyses were made only after removal of the kidney function, for experience relative to the blood sugar content of both normal fasting rabbits and phlorhizinized rabbits made unessential the preliminary determination of the blood sugar content By omission of the estimation of blood sugar content before abolition of the kidney function the well-known influence of the removal of a relatively large quantity of blood upon the percentage of sugar in the blood was obviated

The double ligation of the kidneys in dogs practically amounted to extirpation of these organs and hence was equivalent to the non-participation of the kidneys in the sequence of phenomena following the operation In the case of rabbits, however, the circulation through the kidneys was presumably more or less intact although the secreting mechanism was abolished [7] Any influence exerted by phlorhizin upon the renal organs in the last mentioned instance, such as *production* of sugar, would therefore be possible through the channel of the circulation If the kidney is specifically responsible for the blood sugar phenomena exhibited in phlorhizin diabetes the results obtained from examination of the blood sugar content under the two conditions just outlined should theoretically at least be totally unlike Ligation of the kidneys might be expected under the experimental conditions to maintain the condition of hypoglycaemia or at most to allow blood sugar content to become normal If the kidneys actually *produce* sugar from some antecedent in the blood, as suggested by Pavy, the blood sugar content might be assumed to increase even above the normal by reabsorption in the absence of free secretion, provided it is granted that in the nephritis induced sufficient normal renal cells are present to accomplish such a task On the contrary, if the renal cells after tartrate injection are totally incapacitated from producing sugar under the influence of phlorhizin, that is, practically every cell has lost its function—a condition which is most

[7] Underhill *loc cit*

unlikely—then the blood sugar should behave in the manner indicated for ligation of the kidney, that is, the sugar in the blood should not increase above the normal It has been found that in general *with both types of experiments blood sugar content rose above the normal*

For the establishment of hyperglycaemia under either of the two methods outlined, various explanations may be offered In the first place it may be assumed that phlorhizin acts specifically upon the kidney rendering this organ more permeable for sugar, as suggested by v Mering, and in its attempt to maintain blood sugar content normal the blood sugar regulating mechanism is thrown somewhat out of adjustment, the inhibition is removed or in a manner similar to antibody production there is a compensatory hyperfunction and, in the event of the removal of the kidney function, sugar increases in the blood until hyperglycaemia obtains In the case of rabbits, from the standpoint of Pavy's suggestion, hyperglycaemia could be induced by *production* of sugar in the kidney and reabsorption into the blood Finally, hyperglycaemia may be explained equally well on the assumption that phlorhizin has a two-fold action (a) an influence upon the kidney, resulting in augmented permeability for blood sugar and (b) a *specific* activity upon some other mechanism whereby the organism continually produces new sugar which it throws into the blood stream The latter action might be, however, less pronounced than the former, hence, under ordinary conditions hypoglycaemia is found associated with phlorhizin diabetes The evidence adduced below points in this direction

METHODS Throughout this investigation the experimental animals were maintained in a state of inanition but water was freely given With dogs total phlorhizin diabetes was established according to the procedure recommended by Lusk [8] For the operations, which were performed under aseptic conditions, anaesthesia was produced by ether only After ligation of the kidneys no anaesthetic was necessary for withdrawal of blood samples from a femoral artery Post-mortem examinations demonstrated in each case the complete ligation of the kidneys No attempt was made in the experiments with rabbits to establish a fixed D N

[8] Lusk *Amer Journ of Physiol*, xxii p 163, 1908

TABLE 5

Experiment 3, Rabbit I Female rabbit of 2300 grams received daily subcutaneous injection of 0 25 gram phlorhizin

DATE 1911	URINE			BLOOD		REMARKS
	Volume	Total Nitrogen	Dextrose	Total Solids	Dextrose	
	cc.	*grams*	*grams*	*per cent*	*per cent*	
December						
5	100	2 88	4 03			Water intake = 40 cc
6	200	1 73	3 45			Water intake = 125 cc
7	90	1 84	1 40			Water intake = 50 cc
8	10	0 075	0 076			*Subcutaneous injection of 2 0 grams tartaric acid, neutralized with Na_2Co_3, dissolved in 30 cc water*
9	0			18 0	0 21	Water intake = 150 cc Blood drawn 2 5 hours after last phlorhizin injection The liver contained 0 41 gram glycogen

TABLE 6

Experiment 4, Rabbit J Female rabbit of 2800 grams received daily subcutaneous injection of 0 25 gram phlorhizin

DATE 1911	URINE			BLOOD		REMARKS
	Volume	Total Nitrogen	Dextrose	Total Solids	Dextrose	
	cc	*grams*	*grams*	*per cent*	*per cent*	
December						
5	250	1 45	2 54			Water intake = 170 cc
6	175	2 70	4 46			Water intake = 80 cc
7	125	2 38	2 75			Water intake = 90 cc
8	10	0 075	0 076			*Subcutaneous injection of 2 0 grams tartaric acid, neutralized with Na_2Co_3, dissolved in 30 cc water*
9	0			18 70	0 15	Water intake = 240 cc Blood drawn 2 5 hours after last phlorhizin injection The liver contained 0 60 gram glycogen

dogs the blood sugar content increased above the normal after practical extirpation of the kidneys It is difficult to comprehend how extensive kidney secretory activity could be alleged in the rabbit experiments carried through in the manner described It is evident therefore that under the experimental conditions here outlined little support can be derived in favor of the view advanced by Pavy On the other hand, in view of the new facts furnished by the present investigation the conception of v Mering with respect to the nature of the mechanism of phlorhizin diabetes has been supplemented and extended

TABLE 7

Experiment 6, Rabbit K Female rabbit of 2600 grams received daily subcutaneous injection phlorhizin

DATE 1911	URINE			BLOOD		REMARKS
	Volume	Total Nitrogen	Dextrose	Total Solids	Dextrose	
	cc	*grams*	*grams*	*per cent*	*per cent*	
December						
11	85	1 13	2 40			Water intake = 25 cc Injected 0 25 gram phlorhizin
12	100	1 35	1 58			Water intake = 70 cc Injected 0 25 gm phlorhizin
13	10	0 015	0 008			*Subcutaneous injection of 2 0 grams tartaric acid, neutralized with Na_2Co_3, dissolved in 15 cc water* INJECTED 1 0 GRAM PHLORHIZIN Water intake = 195 cc
14	0			18 65	0 20	Blood drawn 3 5 hours after injection of 1 0 gram phlorhizin The liver was glycogen free

TABLE 8

Experiment 6, Rabbit L Female rabbit of 2800 grams received daily subcutaneous injection of phlorhizin

DATE 1911	URINE Volume			BLOOD Total Solids		REMARKS
	Volume	Total Nitrogen	Dextrose	Total Solids	Dextrose	
	cc	*grams*	*grams*	*per cent*	*per cent*	
December						
11	120	1 71	3 30			Water intake = 50 cc
12	120	2 21	2 01			Water intake = 50 cc
13	2	0	0			*Subcutaneous injection of 3 0 grams tartaric acid, neutralized with Na_2Co_3, dissolved in 20 cc water* INJECTED 1 0 GRAM PHLORHIZIN Water intake = 150 cc
14	0			16 70	0 25	Blood drawn 4 hours after injection of 1 0 gram phlorhizin The liver was glycogen free

SUMMARY

The mechanism of phlorhizin diabetes has been subjected to investigation after the removal of the renal secretory function by (a) ligation of the renal structures in the dog and (b) abolition of kidney secretion through subcutaneous administration of sodium tartrate to rabbits

In both conditions a significant hyperglycaemia may be in evidence With dogs this is accompanied by a decrease in the proportion of solids in the blood, that is, the water content is increased

The data presented lead to the suggestion that phlorhizin may possess a two-fold action (a) an influence is exerted upon the kidney whereby this organ becomes more permeable for blood sugar and (b) an action upon other structures resulting in the production of sugar in quantities sufficient to cause hyperglycaemia if the kidney function is removed

ANIMAL CALORIMETRY

THIRD PAPER

METABOLISM AFTER THE INGESTION OF DEXTROSE AND FAT, INCLUDING THE BEHAVIOR OF WATER, UREA AND SODIUM CHLORIDE SOLUTIONS [1]

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(Received for publication, August 12, 1912)

I INTRODUCTION

The discussion in the last paper of this series noted the fact that, whereas Magnus-Levy found an increase of 20 per cent in the metabolism of a dog during the hours following carbohydrate ingestion, Rubner's work showed an increase of but 5 8 per cent

[1] The urinary analyses of Dog II were accomplished by B Wishart

during a twenty-four-hour period following the ingestion of cane-sugar in such quantity as to furnish energy sufficient to provide for the daily necessities

Heilner[2] repeated Rubner's experiments, using dextrose instead of cane-sugar and found an increase in the heat production equal to 4 per cent in twenty-four hours The animal here employed was kept, as in Rubner's experiments, at an environmental temperature of 33° This work seemed to justify Rubner's conclusion that carbohydrate is a material which may simply replace an isodynamic quantity of fat in metabolism, without materially altering the heat production

Zuntz[3] has criticised Heilner's results on the ground that the respiratory quotient was not determined, and therefore the true conditions were not accurately portrayed In Zuntz's opinion the metabolism was really higher than Heilner calculated

The respiration calorimeter has been used in the following series of experiments to throw additional light upon this problem

II EXPERIMENTAL PART

Experiments were made upon Dog I but still more extensive work was accomplished with Dog II The dogs were both kept at an environmental temperature of between 26° and 27° when within the calorimeter The urine was always free from sugar Other details of the procedure are given in the last paper

A Results of the ingestion of dextrose by Dog I

In the previous paper it was demonstrated that the minimal basal metabolism of Dog I during a morning hour the day after meat had been given at noon was 22 3 calories

Table I of the Appendix presents the metabolism of this dog during the second, third, fourth and fifth hours after the ingestion of 103 grams of dextrose which was taken with 400 cc of water Twenty-four hours before this, the dog had received 200 grams of rice The calculated metabolism rose from a base level of 89 calories for four hours to 115, a difference of 26 calories or 6 5 per

[2] Heilner *Zeitschr f Biol*, l, p 488
[3] Zuntz *Naturwissenschaftliche Rundschau*, xxi, No 38, 1906

hour. This represents an increase of 20 per cent. Since 103 grams of dextrose contain 396 calories and cause an increase in metabolism equal to 26 calories, it follows that 100 calories in dextrose would cause an increase of 6.6 calories. The *non-protein respiratory quotients* were 1.02, 0.99, 0.99 and 0.98 in the successive hours, indicating dominant carbohydrate oxidation.

Of 114.56 calories produced by the animal as calculated from the products of excretion and the oxygen absorption 110.05 calories were measured by the calorimeter. The difference is to be attributed to the greater proportional warming of the skin than of the rectal region, which, in the last paper, was shown to occur in this dog after the ingestion of 100 grams of dextrose. The urine of the period was free from sugar.

This experiment demonstrated without doubt a large rise in the heat production following the ingestion of dextrose by a dog. The respiratory quotients indicate that this increased metabolism could not have been due to the process of converting dextrose into fat.

This completes the record of work to be published with regard to Dog I.

B. The basal metabolism of Dog II.

A variety of problems were investigated upon Dog II. The animal was maintained in good condition and nearly constant weight from November 24, 1911 to May 3, 1912, upon a daily diet consisting of 100 grams of biscuit meal, 100 grams of chopped beef heart meat, 20 grams of fat and 10 grams of bone ash, containing together 5.25 grams of nitrogen and 700 calories. The diet was mixed with water and given to the animal at 6.00 p.m. The *basal metabolism* was determined on the day after the food ingestion usually between the hours of 1.00 p.m. and 4.00 p.m. If the action of a substance were to be determined, it was fed to the dog at noon and the metabolism determined from 1.00 p.m. to 4.00 p.m. or later. If 50 grams of sugar were given at this time, 50 grams of biscuit meal were deducted from the evening ration.

The basal metabolism was found to be the following on the various dates mentioned:

DATE	EXPERIMENT NUMBER	CALORIES		
		Calculated	Found	Average per hour (calculated)
December 2, 1911	3	17 53	17 61	17 53
January 9, 1912	10	15 45	14 54	15 45
January 22, 1912	13	15 41	14 47	
		14 99	17 18	
		30 40	31 65	15 20
January 26, 1912	17	15 73	15 89	
		17 44	16 40	
		15 32	15 42	
		48 49	47 71	16 16
January 30, 1912	18	16 65	14 22	
		15 99	14 98	
		17 96	16 99	
		50 60	46 19	16 86
February 5, 1912	21	15 43	15 11	
		16 03	16 37	
		18 33	17 35	
		49 79	48 83	16 56
April 22 1912	51	14 67	16 10	
		15 22	15 51	
		16 78	17 26	
		46 67	48 87	15 56
Average				16 19

The average heat production of this basal metabolism is 16 2 calories When any three-hour period is compared with this fundamental figure, the average of the three hours is always within 0 7 of a calorie or 4 per cent of the above figure A value which comes between 15 5 and 16 9 calories may therefore be considered as within the limits of the normal basal value of this dog during sleep The full details of these experiments are presented in the Appendix, Table II

Assuming a minimal basal metabolism of 16 2 calories per hour the total would be 388 8 calories in twenty-four hours for a dog weighing 9 3 kgms The area of the dog is 0 4956 square meters calculated from the formula, $11\ 2\ \sqrt[3]{9\ 3^2}$ The minimal metabolism would therefore be 784 calories per square meter of surface, which may be contrasted with 759 calories similarly determined for Dog I The difference is 3 per cent

C The results of the ingestion of dextrose by Dog II

a Twenty grams of dextrose After giving 20 grams of dextrose in 150 cc of water to Dog II at noon, no definite rise in metabolism could be made out in one experiment (No 39) while in a second experiment (No 42) the metabolism rose from the basal value of 16 2 to 18 7 calories in the second hour after the ingestion of the solution The metabolism returned in the third hour to the basal value This rise of 2 5 calories is an increase of 15 per cent above the basal metabolism Since 20 grams of dextrose containing 75 calories causes a rise in heat production of 2 5 calories, it may be estimated that 100 calories would bring about an increase of 3 3 calories

The essential points are here presented and the full details will be found in Table III of the Appendix

EXPERIMENT NUMBER	TIME	NON PROTEIN R Q	CALORIES	
			Calculated	Found
39	1 00-2 00 p m	1 12	16 94	19 12
	2 00-3 00	0 95	16 89	17 24
	3 00-4 00	0 88	15 75	16 55
			49 58	52 92
42	1 00-2 00 p m	1 08	18 72	18 60
	2 00-3 00	0 97	15 43	16 09
			34 15	34 69

It is difficult to explain the high non-protein respira tients It is apparent (Experiment 42) that a small q

dextrose may affect the metabolism during the second hour after its ingestion and then the metabolism reverts to the basal value It is also true that the ingestion of a small amount of dextrose may not increase the metabolism It has been pointed out by Johannson[4] that after giving a small quantity of dextrose (75 grams) to a fasting man, there is no change in the metabolism, on account of the retention of dextrose in the organism in the form of glycogen

b Fifty grams of dextrose The ingestion of 50 grams of dextrose dissolved in 150 cc of water caused a rise in the metabolism from the basal level of 16 2 calories to 19 6 in the second hour, which level was very nearly maintained during the third and fourth hours, and then fell to the basal value in the fifth hour

From the results of four experiments given in detail in Table II of the Appendix, the following type may be taken in illustration

EXPERIMENT NUMBER	TIME	NON PROTEIN R Q	CALORIES Calculated	CALORIES Found
20	1 00-2 00 p m	0 98	19 59	19 94
	2 00-3 00	0 96	19 47	18 45
12	3 00-4 00	1 03	18 23	19 26
	4 00-5 00	0 83	16 61	17 25
			73 90	74 86

It is apparent, from the non-protein respiratory quotients of 98, 96 and 103, that carbohydrate combustion dominated during the second to fourth hours In the fifth hour the quotient fell

The rise from the basal value of 16 2 to 19 6 calories represented an increase of 3 4 calories or 20 per cent The total increase in metabolism during the four hours was 9 1 calories Since 50 grams of dextrose contain 184 6 calories, it follows that, taken in Rubner's sense, the ingestion of 100 calories of dextrose causes an increased metabolism amounting to 4 9 calories

c Seventy-five grams of dextrose Administration of 75 grams of dextrose in 200 cc of water caused an initial rise in metabolism from 16 2 to 19 6 calories, the same as produced by 50 grams of dextrose The increased metabolism was, however, continued

[4] Johannson *Skand Arch f Physiol*, xxi, p 1, 1909

at least through the fifth hour at a level of about 20 calories per hour The total increase during the four hours was 16 35 calories Since 75 grams of dextrose contain 277 calories, it may be calculated that 100 calories of dextrose cause an increase in metabolism equal to 5 9 calories

The following table illustrates these facts, which are set forth in greater detail in the Appendix, Table II

EXPERIMENT NUMBER	TIME	NON PROTEIN R Q	CALORIES	
			Calculated	Found
40	1 00 2 00 p m	1 05	19 60	20 85
	2 00-3 00	1 01	20 22	21 28
	3 00-4 00	0 98	21 09	20 38
	4 00-5 00	1 04	20 24	19 44
			81 15	81 95

The respiratory quotient is such as to indicate oxidation of dextrose throughout the four hours Although the results here presented do not justify the assumption that the higher metabolism terminated with the fifth hour after dextrose ingestion, yet there is indirect evidence which will be offered in the next paper of this series that this is so This evidence consists in the fact that after giving 50 grams of dextrose, a large excretion of water by the kidney marks the fourth hour, the last hour of increased metabolism, whereas after giving 75 grams, the large excretion of water marks the fifth hour

d The nitrogen elimination after dextrose ingestion In the calculations made above, the nitrogen of the urine was collected for the whole period beginning with the catheterization which took place just before placing the animal in the calorimeter and ending with the catheterization after removal of the dog from the box at the end of the experiment, and this nitrogen was apportioned equally between the hourly periods As a matter of fact, however, the nitrogen elimination is far from even after the administration of 50 grams of dextrose, as will be seen from the following analyses

Urinary analyses after giving 50 grams of dextrose at noon

DATE	TIME	TOTAL N	NH₃-N	UREA-N	DATE	TOTAL N
		gram	*gram*	*gram*		*gram*
January 12–'12	11 00–12 00				January 16–'12	0 137
	12 00– 1 00	0 138	0 011	0 101		0 124
	1 00– 2 00	*0 166*	*0 012*	*0 133*		*0 154*
	2 00– 3 00	0 180	0 018	0 156		0 190
	3 00– 4 00	0 264	0 014	0 225		0 181
	4 00– 5 00	0 181	0 011	0 163		0 107

It was incidentally noticed that the volume of urine obtained between 3 00 and 4 00 o'clock was very large in quantity, as compared with all the other hours, and often reached 100 cc This led to the work to be described in the next paper This hour in question was also characterized by a large nitrogen excretion The first hour showed a diminished excretion of nitrogen and a small excretion of water Under these circumstances, it was concluded that the hourly excretion of nitrogen could not be a measure of the protein metabolism of the period but rather an indication of a greater or less secretion of urea through the kidney It was, therefore, deemed nearest correct to evenly distribute through the different hours the total nitrogen elimination for the whole experimental period while the dog was in the calorimeter, in order to obtain a measure of the protein metabolism

D Discussion of the results of dextrose ingestion

Some of the results of the work on metabolism after dextrose ingestion may be seen in the following table

	DEXTROSE	RISE IN METABOLISM IN SECOND HOUR	DURATION OF INCREASED METABOLISM	TOTAL INCREASE IN METABOLISM	100 CALORIES DEXTROSE INCREASES METABOLISM
	grams	*per cent*	*hours*	*calories*	*calories*
Dog I	103	20	5*	26 00	6 6
Dog II	20	15	2	2 50	3 3
	50	20	4	9 10	4 9
	75	20	5*	16 35	5 9

*At least See discussion p 33

Here appears a general confirmation of the work of good authorities The increase in metabolism of 20 per cent found by Magnus-Levy is verified, and a "specific dynamic" action of dextrose of 4 9 after giving 50 grams of dextrose is in accord with Rubner's experiments with cane-sugar Rubner's theory was that the extra heat produced by cane-sugar is derived from a cleavage of cane-sugar into levulose and dextrose His idea calls for the absorption of dextrose and its utilization in isodynamic replacement of fat in the metabolism However, this does not occur *During the first four and five hours after the ingestion of 50, 75 and 100 grams of dextrose, the heat production rises 20 per cent above the basal metabolism* This fact was lost to sight in experiments continued over a period of twenty-four hours

What then is the cause of the increased metabolism? Dextrose itself is soluble, ready for absorption and ready for oxidation by the cells Johannson has already been quoted as having shown the absorption of 75 grams of dextrose by a fasting man who retained it as glycogen and no increase in metabolism followed The same writer[5] has given to a diabetic 50 grams of dextrose which was excreted unchanged in the urine and caused no change in the metabolism The question of "Darmarbeit" may, therefore, be excluded as a factor to be considered

Two causes might contribute to the increased metabolism

1 The sugar entering the blood stream might set up osmotic changes which would stimulate the cells to increased movement and, therefore, increase the metabolism

2 The larger quantity of dextrose brought by the blood might cause increased oxidation through the presence of an increased amount of a readily oxidizable food-stuff

To investigate the first problem it was decided to test the metabolism of the dog by giving (1) 200 cc of water, (2) 150 cc of water containing 6 7 grams of sodium chloride, (3) 150 cc of water containing 17 grams of urea The solutions of salt (assuming complete electrolytic dissociation) and of urea were isotonic with 50 grams of dextrose in 150 cc of water

To test the second problem another food-stuff, olive oil in fine emulsion, was given to the dog

[5] Johannson *loc cit*

E The ingestion of water

That water in moderate quantities has no effect on metabolism has already been set forth by Bidder and Schmidt,[6] by Rubner[7] and by Heilner [8] The following results were obtained after giving 200 cc of water at noon (Appendix, Table I) and also, for comparison, the results of the day following when no food was given at noon (Appendix, Table II) The dog was given the regular standard diet at 6 00 p m as in all other experiments

Experiment 16—200 cc of water, January 25, '12

TIME	CALORIES	
	Calculated	Found
1 00–2 00 p m	16 52	17 22
2 00–3 00	16 52	17 69
3 00–4 00	16 46	17 34
	49 50	52 25

Experiment 17—No food, January 26, '12

TIME	CALORIES	
	Calculated	Found
1 00–2 00 p m	15 73	15 89
2 00–3 00	17 44	16 40
3 00–4 00	15 32	15 42
	47 71	48 49

It is evident that the ingestion of that quantity of water in which the dextrose given in the experiments was dissolved, can have no influence on the metabolism

F The ingestion of urea in solution

In Experiment 14, 17 grams of urea in 150 cc of water were given the dog at noon and the animal was placed in the calorimeter At 12 45 this was largely vomited The experiment was continued for two hours, although the measurement by the calorimeter during the first hour is not to be relied upon, on account of the mass of water on the floor of the box

In Experiment 19, 12 grams of urea were given, which was properly retained by the animal The results of these experiments are presented below (full details, Appendix, Table II)

[6] Bidder and Schmidt *Verdauungssäfte und Stoffwechsel*, 1852, p 340
[7] Rubner *Gesetze des Energieverbrauchs*, 1902, p 62
[8] Heilner *Zeitschr f Biol*, xlix, p 373, 1907

TIME	*Experiment 14—17 grams (?) urea, 150 cc water, at noon* CALORIES		*Experiment 19—12 grams urea, 150 cc water, at noon* CALORIES	
	Calculated	Found	Calculated	Found
1 00-2 00 p m	15 85	20 12 (?)		
2 00-3 00	17 76	18 76	16 35	15 94
3 00-4 00			16 68	16 07
	33 61		33 03	32 01

In Experiment 14, during the period before the dog was put into the calorimeter, the nitrogen eliminated amounted to 0 154 gram hourly, whereas, after administration of the urea solution, it rose to 0 424 gram per hour, indicating a considerable absorption of urea In the successful Experiment 19, the hourly excretion of nitrogen rose from 0 133 gram before the ingestion of urea to 0 602 gram after, which corresponds to the elimination of an extra gram of urea per hour As the results show, *the elimination of urea after its administration in solution is without any influence upon metabolism* The normal of 16 2 calories per hour was closely approximated

Zuntz[9] has sought to attribute part of the "specific dynamic" action of protein to the increased kidney activity accompanying the elimination of urea, but the above experiment shows this to be without foundation

The calculation of the protein metabolism in Experiment 14 and Experiment 19 was on the assumption that it was maintained at the same height as on near-by days when no food was given during the same interval of the day

G *The ingestion of sodium chloride in solution*

One experiment was made to determine the influence of the ingestion of 6 7 grams of sodium chloride in 150 cc of water, a solution which is isotonic with 50 grams of dextrose in 150 cc of water This was given at noon on two successive days, the metabolism being obtained on the second day with the following results (details, Appendix, Table II)

[9] Zuntz *Zentralbl f Physiol*, xxiii, p 960, 1909

Experiment 15—6 7 grams NaCl in 150 cc of water

TIME	CALORIES	
	Calculated	Found
1 00-2 00 p m	15 09	18 00
2 00 3 00	16 64	17 37
3 00-4 00	16 19	16 54
	47 92	51 91

These figures contradict the statement of Zuntz[10] regarding the behavior of sodium chloride on metabolism The results in no way differ from the average basal metabolism of 16 2 calories per hour Hence, *the ingestion of 150 cc of a solution containing 4 2 per cent sodium chloride is without influence upon the metabolism*

The urinary excretion of sodium chloride during the experimental period between 12 00 noon and 4 08 p m amounted to 0 805 gram per hour and from 4 08 to 10 a m the next day to 0 439 gram per hour The quantity of urine was 140 cc during the experimental period of four hours, indicating marked diuresis

H The ingestion of fat

Since dextrose solutions caused an increased metabolism and solutions such as 8 per cent urea and 4 2 per cent sodium chloride and water itself had no effect, it seemed desirable to test the influence of another food-stuff, fat, which is quickly absorbed, enters the blood current and supplies the cells with nutriment

Fifty grams of olive oil were mixed with 10 cc of a 1 2 per cent sodium carbonate solution and shaken till a fine emulsion formed, and this was given to the dog at noon

The results may be thus summarized (details, Appendix, Table II)

	Experiment 23—50 grams olive oil, at noon		*Experiment 24—50 grams, olive oil, at noon*	
TIME	CALORIES		CALORIES	
	Calculated	Found	Calculated	Found
1 00-2 00 p m		18 71	17 69	19 77
2 00-3 00		18 05	19 71	19 73
		36 76	37 40	39 50

Oxygen lost but the CO_2 per hour was 6 05 and 5 91 against 5 81 in experiment 24 on day following during which hour the calculated metabolism was 17 69 the R Q being 0 79 Given these latter conditions the calculated metabolism of experiment 23 would have been 18 calories and over in the two hours recorded

[10] Zuntz *Zentralbl f Physiol*, xxiii, p 960, 1909

These experiments illustrate an increased metabolism above the basal level of 16 2 calories per hour, due to the inflow of fat If one again recalls Benedict's experiments showing the negative effect of cathartics and of agar-agar upon the metabolism, one cannot attribute this increase to "Darmarbeit " The emulsified fat flows partly through the portal vein, but in large quantity enters directly into the circulation through the thoracic duct, at once affording a means of enrichment of the fat content of the general circulation Under these circumstances of ample nutrition, the metabolism increases (in the sense of Voit)

The general results obtained from the experiments mentioned in this paper are shown in the accompanying chart

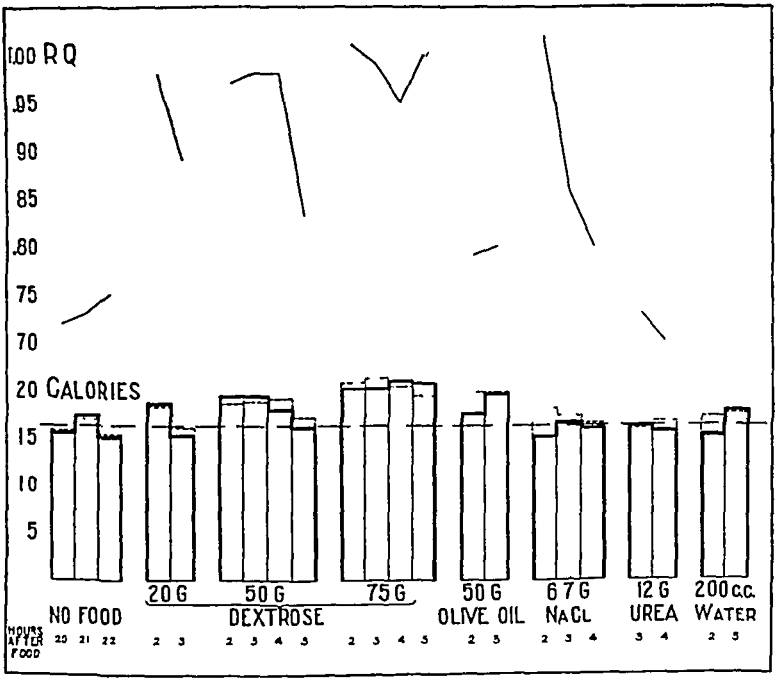

CHART 1 ILLUSTRATING THE EFFECT OF THE INGESTION OF DEXTROSE AND FAT AND OF WATER, UREA AND SALT SOLUTIONS ON THE METABOLISM

Solid lines—metabolism in calories as calculated Broken lines—metabolism in calories as found

III SUMMARY

It is impossible to discuss the present work without anticipatory reference to the results of Miss Fisher and Miss Wishart, which are to be presented in the paper which follows this These authors have found that after giving 50 grams of dextrose to a dog, the blood sugar rises in percentage within one hour and then falls to normal during the succeeding hours, that there is a large retention of water by the organism during the period of high metabolism, which water is suddenly eliminated during the last hour of the high metabolism (the fourth after dextrose ingestion), that during this same hour, the absorption of dextrose is completed, and that, during the period of high metabolism and water retention, the percentage of hemoglobin falls in the blood, rising again after the water elimination

In general, the following picture may be drawn It was noted in Dog I, that, after giving 100 grams of dextrose, the skin temperature rose during the second hour, while the rectal temperature fell slightly (second paper) When 50 grams of dextrose are given (Dog II) the sugar content of the blood in per cent is first increased, but by the end of the second hour and thereafter, it is found to have returned to its normal percentage value The volume of the blood, however, increases during the second hour, obviously in response to the osmotic pressure exerted by the increased sugar concentration The increase in blood volume is demonstrated by the fall in the hemoglobin content of the blood During this period, the metabolism shows an increase of 20 per cent above the normal basal value During the fourth hour there is final absorption of all the dextrose ingested, the metabolism remains high and there is a large excretion of water in the urine At the end of the same fourth hour, the blood sugar content is normal, indicating that, during the withdrawal of water and the concentration of the blood, the liver and muscles maintain the normal sugar content of the blood During the second, third and fourth hours, the high metabolism is accompanied by respiratory quotients of about 1 00 (after allowing for the influence of protein metabolism) However, during the fifth hour, when the metabolism has returned to its basal value, a non-protein respiratory quotient of 0 83 indicates that a mixture of fat and carbohydrate is being oxidized The glycogenic function apparently enters into control and reduces the amount of carbohydrate available for combustion, and fat is oxidized as well

It is interesting to recall in this connection that Hári[11] found that the respiratory quotient was increased in a fasting animal after giving adrenalin Adrenalin causes a discharge of sugar from the glycogen repositories of the organism,[12] the percentage of blood sugar rises largely and, in virtue of this, carbohydrate tends to replace fat in the oxidative processes

If 20 grams of dextrose be given, a rise of 15 per cent may take place during the second hour after sugar ingestion, to be followed by a normal basal metabolism in subsequent hours

If 75 grams of dextrose be given, a 20 per cent increase in metabolism continues from the second through the fifth hour, during which hour the volume of urine becomes large, indicating that this is the final hour of the higher metabolism

It is shown that ingestion of 200 cc of water, of 150 cc of a 4 2 per cent solution of sodium chloride or of 150 cc of an 8 per cent solution of urea have no influence upon metabolism It is, therefore, concluded that the high metabolism after giving dextrose is not due to osmotic changes between the blood and the tissues The only remaining conclusion which appears possible is that *the increase in metabolism is due to the presence of a greater amount of free diffusible carbohydrate than is present when there is no absorption of carbohydrate from the intestines* It is known that the sugar of the blood behaves as if it existed in chemical combination (Loewi, Lépine) If this combining power over sugar remains constant, then when the blood is diluted with coincident maintenance of the normal percentage content of dextrose, there would be an increased supply of free readily oxidizable sugar molecules available for the nutrition of the cells Hence, carbohydrate alone is oxidized, and the metabolism rises in virtue of an increased supply of nutriment

An emulsion of olive oil ingested by the dog caused an increase in metabolism, probably for similar reasons

These experiments confirm the work of Zuntz and of Rubner in many particulars, but they bring to light new points which show that the theories of both Zuntz and Rubner will have to be revised

The views here expressed, however, conform to the "older" view of Carl Voit, who believed that the presence of abundant food increased the power of the cells to metabolize

[11] Hári *Biochem Zeitschr*, xxxviii, p 23, 1912
[12] Ringer *Journ of Exp Med*, xii, p 105, 1910

APPE

TABLE I

DATE	TIME	EXP NO	CO_2	O_2	R Q	H_2O	URINE N	NON PROTEIN CO_2	NON PROTEIN O_2	NON PROTEIN R Q
			grams	grams		grams	grams	grams	grams	
May 4, '11	10 00–11 00 a m	34	10 85	7 94	0 99	9 27	0 213	8 86	6 14	1 02
	11 00–12 00		10 68	8 15	0 95	9 06	0 213	8 65	6 35	0 99
	12 00–1 00 p m		11 30	8 59	0 96	9 28	0 213	9 28	6 79	0 99
	1 00–2 00		11 23	8 64	0 95	9 31	0 213	9 20	6 84	0 98

TABLE II

DATE	TIME	EXP NO	CO_2	O_2	R Q	H_2O	URINE N	NON PROTEIN CO_2	NON PROTEIN O_2	NON PROTEIN R Q
			grams	grams		grams	grams	grams	grams	
December 2 11	9 57–10 57 a m	3	5 61	5 21	0 76	9 87	0 133	4 03	4 09	0 72
January 9, 12	10 00 11 00 a m	10	5 40	4 72	0 83	9 41	0 1331	4 14	3 50	0 86
January 22 12	1 00–2 00 p m	13	5 56	4 55	0 89	8 66	0 124	4 40	3 50	0 92
	2 00–3 00		5 26	4 47	0 86	8 25	0 124	4 11	3 42	0 87
January 26 12	1 00–2 00 p m	17	4 75	4 83	0 72	7 31	0 095	3 89	4 03	0 70
	2 00–3 00		5 26	5 35	0 73	7 50	0 095	4 40	4 55	0 70
	3 00–4 00		4 84	4 67	0 75	7 23	0 095	3 96	3 87	0 74
January 30 12	1 00–2 00 p m	18	5 14	5 12	0 73	8 60	0 130	3 92	4 02	0 71
	2 00–3 00		5 14	4 78	0 78	7 97	0 130	3 92	3 78	0 75
	3 00–4 00		5 53	5 52	0 73	8 03	0 130	4 33	4 42	0 71
February 5 12	1 00–2 00 p m	21	5 32	4 60	0 84	6 51	0 094	4 44	3 80	0 85
	2 00–3 00		5 11	4 88	0 76	6 27	0 094	4 22	4 08	0 75
	3 00–4 00		5 61	5 61	0 73	6 54	0 094	4 73	4 81	0 72
April 22, '12	12 00–1 00 p m	51	5 03	4 38	0 84	10 29	0 132	3 80	3 26	0 85
	1 00–2 00		4 98	4 60	0 79	8 99	0 132	3 75	3 48	0 78
	2 00–3 00		5 23	5 12	0 74	8 18	0 132	4 00	4 00	0 73

In experiments 3 and 10 standard diet was given at noon in the others at 6 p m daily

DOG I

Calories: Protein	Calories: Non Protein	Calories: Total Calculated	Calories: Total Found	Body Temperature: Start	Body Temperature: End	Body Temperature: Difference	Morning Weight	Food
							Kilograms	
5 64	21 70	27 34	26 04	37 82	38 15	+0 33	16 3	103 grams dextrose + 400 cc water at 9 15 a m
5 64	22 42	28 06	26 42		38 19	+0 04		
5 64	24 03	29 67	26 22		38 08	−0 11		
5 64	23 85	29 49	31 37		38 45	+0 37		
		114 56	110 05					

DOG II

Calories: Protein	Calories: Non Protein	Calories: Total Calculated	Calories: Total Found	Body Temperature: Start	Body Temperature: End	Body Temperature: Difference	Morning Weight	Behavior of Dog	Remarks
							kg		
4 07	13 46	17 53	17 61	37 89	37 85	−0 04	9 3	Very quiet	No food
3 51	11 94	15 45	11 54	38 41	38 41		8 9	Quiet	No food
3 29	12 12	15 41	14 47	38 66	38 45	−0 21	8 7	Very quiet	No food
3 29	11 70	14 99	17 18		38 48	+0 03		Active 7 minutes O_2 low	
		30 40	31 65						
2 52	13 21	15 73	15 89	38 36	38 26	−0 10	8 8	Very quiet	No food
2 52	14 92	17 44	16 40		38 27	+0 01		Slight movements	
2 52	12 80	15 32	15 42		38 22	−0 05		Moving 1 minute	
		48 49	47 71						
3 45	13 20	16 65	14 22	38 43	38 32	−0 11	8 8	Quiet	No food
3 45	12 54	15 99	14 98		38 28	−0 04		Occasional movement	
3 45	14 51	17 96	16 99		38 43	+0 15		Active 1 minute	
		50 60	46 19						
2 50	12 93	15 43	15 11	38 40	38 31	−0 09	9 0	Very quiet	No food
2 50	13 53	16 03	16 37		38 44	+0 13		Occasional movements	
2 50	15 83	18 33	17 35		38 62	+0 18		Active 2 minutes	
		49 79	48 83						
3 58	11 09	14 67	16 10	37 59	37 58	−0 01	9 4	Very quiet	No food
3 58	11 64	15 22	15 51		37 51	−0 07		Very quiet	
3 58	13 20	16 78	17 26		37 64	+0 13		Quiet	
		46 67	48 87						

TABLE II

DATE	TIME	EXP NO	CO_2	O_2	R Q	H_2O	URINE N	NON PROTEIN CO_2	NON PROTEIN O_2	NON PROTEIN R Q
			grams	*grams*		*grams*	*grams*	*grams*	*grams*	
March 26, 12	1 00 2 00 p m	39	6 98	4 87	1 04	7 11	0 150	5 58	3 60	1 12
	2 00 3 00		6 22	4 97	0 91	7 33	0 150	4 82	3 70	0 95
	3 00-4 00		5 49	4 72	0 84	7 04	0 150	4 09	3 45	0 86
March 30 12	1 00-2 00 p m	42	7 46	5 54	0 98	9 08	0 200	5 59	3 75	1 08
	2 00-3 00		5 74	4 68	0 89	8 63	0 200	3 87	2 89	0 97
January 3 12	10 20-11 20 a m	9	5 27	4 47	0 86	9 08	0 161	3 78	3 10	0 89
	1 00-2 00 p m		7 69	5 77	0 97	10 76	0 136	6 42	4 62	1 01
	2 00-3 00		8 34	6 76	0 90	10 89	0 136	7 04	5 61	0 91
	3 00-4 00		9 63	7 59	0 92	12 51	0 136	8 36	6 44	0 94
	4 00-5 00		6 27	5 02	0 91	10 30	0 136	4 99	3 87	0 94
January 12 12	1 00-2 00 p m	11	7 59	5 70	0 97	8 71	0 143	6 23	4 49	1 00
	2 00-3 00		7 65	5 69	0 98	8 28	0 143	6 31	4 48	1 01
January 18 12	3 00-4 00 p m	12	7 10	5 27	0 98	9 22	0 133	5 87	4 15	1 03
	4 00-5 00		5 71	4 99	0 83	8 62	0 133	4 44	3 87	0 83
February 2, '12	1 00-2 00 p m	20	7 41	5 71	0 94	8 41	0 150	6 01	4 44	0 98
	2 00-3 00		7 25	5 70	0 93	8 49	0 150	5 87	4 43	0 96
March 27 12	1 00-2 00 p m	40	7 83	5 66	1 01	8 19	0 115	6 76	4 69	1 05
	2 00-3 00		7 82	5 79	0 99	7 91	0 115	6 75	4 82	1 01
	3 00-4 00		8 01	6 14	0 95	8 39	0 115	6 94	5 17	0 98
	4 00-5 00		8 07	5 84	1 00	7 99	0 115	7 00	4 87	1 04
January 25 12	1 00-2 00 p m	16	5 31	4 60	0 84	7 63	0 130	4 11	*3 50	0 85†
	2 00-3 00		5 13	5 51	0 68	7 66	0 130	3 92	*4 41	0 65†
	3 00-4 00		5 31	5 01	0 77	7 42	0 130	4 11	3 91	0 76

* Average 3 95 † Average 74

(Continued)

Calories				Body temperature			Morning weight	Behavior of dog	Remarks
Protein	Non Protein	Total Calculated	Total Found	Start	End	Difference			
							kg		
3 98	12 96	16 94	*19 12	37 94	38 22	+0 28	9 3	Very quiet	20 grams dextrose in 150 cc water at noon
3 98	12 91	16 89	17 24		38 10	−0 06		Quiet	
3 98	11 77	15 75	16 56		38 04	−0 12		Quiet	
		49 58	52 92						
5 30	13 42	18 72	18 60	38 20	38 32	+0 12	9 4	Very quiet	20 grams dextrose in 150 cc water at noon
5 30	10 13	15 43	16 09		38 20	−0 12		Very quiet	
		34 15	34 69						
4 29	10 66	14 9[illegible]	14 13	38 03	38 01	−0 02	8 5	Quiet	50 grams dextrose in 150 cc of water at noon
3 62	16 32	19 94	19 86	38 69	38 72	+0 03		Active 2 minutes	
3 62	19 39	23 01	21 78		38 92	+0 20		Active 10 minutes	
3 62	22 41	26 03	24 06		38 85	−0 07		Active 23 minutes	
3 62	13 47	17 09	17 12		38 62	−0 23		Quiet	
		101 02	106 95						
3 80	15 86	19 66	18 69	38 59	38 81	+0 22	8 5	Quiet	
3 80	15 83	19 63	18 83		39 05	+0 24		Quiet	
		39 29	37 52						
3 51	14 72	18 23	19 26	38 61	38 69	+0 08	8 5	Very quiet	
3 51	13 10	16 61	17 25		38 59	−0 10		Moving 2 minutes	
		34 84	36 51						
3 98	15 61	19 59	19 90	38 59	38 88	+0 29	8 6	Very quiet	
3 98	15 49	19 47	18 45		38 84	−0 04		Very quiet	
		39 06	38 35						
2 91	16 69	19 60	20 85	37 07	38 24	+0 27	9 3	Very quiet	75 grams dextrose in 200 cc of water
2 91	17 31	20 22	21 28		38 42	+0 18		Restless 3 minutes	
2 91	18 18	21 09	20 38		38 25	−0 17		Very quiet	
2 91	17 33	20 24	19 44		38 33	+0 08		Quiet	
		81 15	81 95						
3 45	13 07	16 52	17 22	38 73	38 64	−0 09	8 8	Very quiet	200 cc of water
3 45	13 07	16 52	17 69		38 61	−0 03		Active 1 minute	
3 45	13 01	16 46	17 34		38 69	+0 08		Quiet	
		49 50	52 25						

*Heat eliminated = 17.01

TABLE II

DATE	TIME	EXP NO	CO_2	O_2	R Q	H_2O	URINE N	NON PROTEIN CO_2	NON PROTEIN O_2	NON PROTEIN R Q
			grams	grams		grams	grams	grams	grams	
January 23 '12	1 00-2 00 p m	14	5 86	4 65	0 92	19 48	‡0 124	4 69	3 60	0 95
	2 00-3 00		5 80	5 39	0 78	19 21	0 124	4 62	4 34	0 77
February 1 '12	2 00-3 00 p m	19	5 06	5 03	0 73	8 15	‡0 130	3 85	3 93	0 71
	3 00-4 00		5 07	5 23	0 70	7 64	0 130	3 85	4 13	0 68
January 24, '12	1 00- 2 00 p m	15	6 00	4 29	1 02	8 86	0 219	3 96	2 44	1 18
	2 00- 3 00		5 92	5 00	0 86	8 94	0 219	3 85	3 15	0 89
	3 00- 4 00		5 45	4 94	0 80	8 13	0 219	3 41	3 09	0 80
February 7 12	1 00- 2 00 p m	23	6 05	lost		7 49	0 139			
	2 00- 3 00		5 91	lost		7 69	0 139			
February 8 12	1 00- 2 00 p m	24	5 81	5 36	0 79	7 17	0 129	4 58	4 27	0 78
	2 00- 3 00		6 61	6 03	0 80	7 43	0 129	5 39	4 94	0 79

‡ See text

(Continued)

Calories				Body Temperature			Morning Weight	Behavior of Dog	Remarks
Protein	Non Protein	Total Calculated	Total Found	Start	End	Difference			
							kg		
3 29	12 56	15 85	20 42	38 63	38 41	−0 22	8 7	Very quiet	17 grams urea in 150 cc water (vomited)
3 29	14 47	17 76	18 75		38 16	−0 25		Active 5 minutes	
		33 61	39 17						
3 45	12 90	16 35	15 94	39 02	38 71	−0 31	8 6	Very quiet	12 grams urea in 150 cc water
3 45	13 23	16 68	16 07		38 55	−0 16		Very quiet	
		33 03	32 01						
5 81	9 28	15 09	18 00	38 78	38 70	−0 08	8 7	Quiet	6 7 gms NaCl in 150 cc water
5 81	10 83	16 64	17 37		38 64	−0 06		Active 2 minutes	
5 81	10 38	16 19	16 54		38 62	−0 02		Quiet	
		47 92	51 91						
			18 71	38 59	38 80	+0 21		Very quiet	50 grams olive oil
			18 05		38 84	+0 04		Very quiet	
			36 76						
3 42	14 27	17 69	19 77	38 48	38 76	+0 28		Quiet	50 grams olive oil
3 42	16 29	19 71	19 73		39 04	+0 28		Quiet	
		37 40	39 50						

ANIMAL CALORIMETRY

FOURTH PAPER

OBSERVATIONS ON THE ABSORPTION OF DEXTROSE AND THE EFFECT IT HAS UPON THE COMPOSITION OF THE BLOOD

By GERTRUDE FISHER AND MARY B WISHART

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(Received for publication, August 12, 1912)

In the experiments described in the last paper, two results were noted In the first place, the temperature of the skin rose to a greater degree after giving dextrose than did the rectal temperature, and in the second place, the period of high metabolism was terminated by an hour in which a large quantity of water was eliminated in the urine The first observation pointed to a change in the distribution of the blood, whereas the second indicated that the volume of the blood had been increased during the period of the higher metabolism, only to diminish again through the sudden excretion of water at the end of the period This paper presents results designed to follow these facts to a definite interpretation

An old observation of Bischoff and Voit[1] noted that a dog which had been fed with bread for forty-one days, during which time the urinary nitrogen indicated a loss of 3 7 kgms of "flesh," in reality lost only 0 5 kgm in weight, and when 1800 grams of meat were given to the animal, water was eliminated in larger quantity in the urine than was taken in the food Although the fact of an increased quantity of water held by the tissues when an animal is maintained on a carbohydrate diet, has been repeatedly confirmed, there has been no analysis of this condition extending over a short period of time

[1] Bischoff and Voit *Die Gesetze der Ernährung des Fleischfressers*, 1860, p 211

Experiments which describe the effect of dextrose ingestion upon the content of blood sugar have been accomplished on man Recent determinations by E Frank[2] give values of 0 07 to 0 09 per cent of dextrose in normal human blood Liefmann and Stern[3] state that 0 105 per cent represents the maximum amount of dextrose normally present However, these authors note that in two patients with croupous pneumonia, the dextrose content of the blood was 0 108 and 0 136 per cent, which, however, rose one hour after the ingestion of 200 grams of dextrose to 0 17 and 0 281 per cent respectively Gilbert and Baudouin[4] have given 150 grams of dextrose in 400 cc of water to six normal young men They report that the blood sugar rose after one hour and amounted to 0 107, 0 108, 0 125, 0 130, 0 132 and 0 134 per cent in the six individuals After two hours the blood sugar content was only slightly above the normal, and in the third and fourth hours it was once more entirely normal

In addition to these experiments, the procedure in which is entirely comparable to that employed by the present writers, other experiments have been performed in which dextrose solutions were injected directly into the venous system and the results noted Thus Brasol,[5] in the laboratory of Carl Ludwig, found that two minutes after intravenous injection of dextrose solutions, much of the injected dextrose was already removed from the blood, the percentage of hemoglobin fell, indicating a dilution due to osmotic processes, but in two hours the normal percentage of sugar had been reached Repeating these experiments, Nadporozsky[6] found that, following intravenous injection of dextrose solutions, the hemoglobin at first fell but after forty-five minutes exhibited normal relations Starling[7] injected 40 grams of dextrose in 40 cc of water into the jugular vein of a dog Within five minutes the hemoglobin in the blood fell from 100 to 60 per cent but after half an hour returned to normal Accompanying the hydremic

[2] E Frank *Zeitschr f physiol Chem*, lxx, p 139, 1911

[3] Liefmann and Stern *Biochem Zeitschr*, i, p 301, 1906

[4] Gilbert and Baudouin *Compt rend soc biol*, lxv, p 710, 1908

[5] Brasol *Arch f Physiol*, 1884, p 211

[6] Nadporozsky *Ruskaja Medicina*, 1887, No 26 Abstract in *Maly's Jahresbericht für Tierchemie*, 1887

[7] Starling *Journ of Physiol*, xxiv, p 317, 1899, see also *The Fluids of the Body*, 1909

plethora, due to the withdrawal of water from the tissues in consequence of osmotic differences, there was a rise in blood pressure, an increase in kidney volume and an increase in the elimination of urine Starling[8] believes that the consequences of the hydremic plethora are increased circulation through the kidney and increased output of urine, the dilution of the blood favoring glomerular filtration The urine contained dextrose and Starling also suggests that dextrose itself leads to a stimulus of the kidney vessels or possibly the kidney cells, because the diuresis outlasts the hydremic plethora During this latter period, the quantity of hemoglobin rises above the normal on account of the continued diuresis

Biedl and Kraus[9] have administered intravenously, 20 and 30 grams of dextrose in 10 per cent solutions to human beings, have noted a rapid disappearance of excess of sugar in the blood, and have found that no considerable amount of sugar was eliminated in the urine These experiments all point to the efficiency of the glycogenic function of the body which tends to maintain the dextrose content of the blood a fixed level

EXPERIMENTAL PART

In paper three was described how a dog (Dog II), weighing about 9 kgms, was maintained on a standard diet and how eighteen hours after food ingestion, dextrose was administered (usually in 150 cc of water) and the metabolism then determined in hourly periods The following experiments were designed to nearly reproduce these conditions with a view to ascertaining certain details during the different hours of dextrose absorption The facts investigated were (a) the amount of dextrose in the gastro-intestinal tract, (b) the dextrose content of the blood, (c) the glycogen content of the liver, (d) the quantity of urine secreted and (e) the hemoglobin content of the blood The first three factors were determined upon ten different dogs (shown in Table I) while the last two factors were observed upon the same dog (shown in Table II)

Reference to Table I reveals a series of experiments upon ten dogs which were given a standard diet one or more days In all

[8] Starling *The Fluids of the Body*, 1909, p 153

[9] Biedl and Kraus *Wiener klin Wochenschr*, ix p 55

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the cases blood was withdrawn under cocaine anesthesia, a method which was employed because most anesthetics lead to an output of dextrose in the urine The cocaine was first used in a 7 per cent solution, which proved too strong and led to the death of the animal in convulsions With a 1 per cent solution of cocaine there was no difficulty throughout the entire series of experiments, except in the case of a small nervous dog (IV) In the other instances, after the subcutaneous administration of cocaine immediately over the carotid artery, a canula could be inserted in the vessel and two samples of blood of about 50 cc each could be drawn from the perfectly quiet animal without any sign of pain or irritation The dog was then killed by a sudden blow on the head, the liver quickly removed and the stomach and intestinal contents were covered with 95 per cent alcohol

The blood sugar was determined by the method of Weymouth Reid[10] as used by Vosburgh and Richards [11] Glycogen in the liver was determined according to Pfluger[12] and all sugar determinations according to the method of Allihn The Fleischl-Miescher method was employed in the hemoglobin tests

a The quantity of dextrose in the gastro-intestinal tract

In five cases, when the animal was killed twenty-four hours after administration of the standard diet, the gastro-intestinal tract was found to be free from dextrose When 50 grams of dextrose were given about twenty-four hours after the standard diet, a rapid though variable absorption of dextrose was recorded The results may thus be summarized

Rate of absorption after giving 50 grams of dextrose

TIME AFTER FOOD	DEXTROSE	
	In stomach	In intestine
	grams	*grams*
One hour	5 6	2 9
Two hours	11 1	1 7
Two hours	16 6	1 0
Three hours	7 4	0 0
Four hours	0 0	0 0

[10] Reid *Journ of Physiol*, xx, p 316, 1896
[11] Vosburgh and Richards *Amer Journ of Physiol*, ix, p 35, 1903
[12] Pflüger *Das Glycogen,* 2te Aufl, 1905 p 67

These figures indicate that the absorption of 50 grams of dextrose in dogs weighing between 8 and 9 kgms is rapid and is completed during the fourth hour after the administration of the sugar

b The dextrose content of the blood

It has been repeatedly verified that convulsions increase the quantity of blood sugar through depletion of the glycogen reserves of the body This is apparent in values of 0 19 and 0 14 per cent of dextrose contained in the blood taken during cocaine convulsions In two cases, however, when the blood was obtained from a quiet dog twenty-four hours after food ingestion, duplicate analyses showed in one dog 0 10 and in another 0 11 per cent of glucose, which are entirely normal values

After giving 50 grams of dextrose in 150 cc of water to dogs which had received the standard diet twenty-four hours before, the following results were obtained

Time after 50 grams dextrose	Blood sugar in per cent
One hour	0 16
*One hour	0 13
Two hours	0 10
Two hours	0 11
Three hours	0 11
Four hours	0 11

*Not included in Table I

These results confirm those of Gilbert and Baudouin on man, in showing that the blood sugar rises during the first hour and then falls, remaining normal thereafter

c The glycogen content of the liver

The glycogen content of the liver is known to be very variable in different dogs twenty-four hours after food ingestion, so that glycogen determinations often show little that is significant In this series, in the two "normal" cases, there were 7 47 grams (2 97 per cent) and 7 70 grams (2 7 per cent) of glycogen in the liver Much less was found in the dogs which were killed during convulsions, being 3 26, 5 81 and 2 28 grams (2 00, 1 48 and 1 31 per cent)

The following table shows the quantity of glycogen found in the liver at different hours after giving 50 grams of dextrose, and

this is compared with the quantity of dextrose absorbed from the intestine as actually determined

The influence of ingesting 50 grams of dextrose on the glycogen content of the liver

TIME AFTER 50 GRAMS OF DEXTROSE	DEXTROSE INGESTED		LIVER GLYCOGEN	
	Absorbed	Present in body but unoxidized (calculated)		
	grams	*grams*	*grams*	*per cent*
One hour	11 5	37 1	12 75	3 33
Two hours	37 2	28 4	7 95	3 30
Two hours	32 4	23 6	11 90	3 85
Three hours	12 6	29 0	6 21	2 56
Four hours	50 0	32 4	21 28	7 24

The respiration experiments in the last paper showed that about 16 calories of carbohydrate were oxidized per hour during the second and third and fourth hours in a dog similar in size to those here employed This is the equivalent of 4 4 grams of dextrose per hour This must be considered a minimal value as it was obtained in a dog in complete repose Adopting this value for all the different periods described above, it is possible to estimate the maximal quantity of the absorbed dextrose which could have been present and unoxidized in the organism at the end of any given experiment

One point stands out clearly and that is that during the period of high metabolism dextrose is not retained in the liver in any considerable quantity The larger part of the absorbed dextrose passes to other tissues Only at the end of four hours and after complete absorption of all the dextrose from the organism, does glycogen appear in large quantity in the liver This fourth hour is the last hour of the high metabolism While it is realized that it is dangerous to draw too sweeping conclusions from a single experiment, still it seems very probable that the end of the fourth hour is marked by a reduction of the flood tide of carbohydrate material to the tissues, the excess being deposited in the liver

d The quantity of urine secreted

In paper three of this series, it was noticed that after giving 50 grams of dextrose in solution to a dog, there was a large secretion of urine during the fourth hour, which terminated the period of high metabolism In the present series of experiments, a dog was used weighing between 7 and 8 kgms The animal was first starved for two days, then on a third day of fasting received 150 cc of water and on the evening of that day and thereafter received the standard diet On various occasions at noon, he received 20 grams and 50 grams of dextrose dissolved in 150 cc of water and 75 grams of dextrose in 200 cc of water These experiments were designed to reproduce the conditions described in paper three

The urine was obtained by means of a sterile catheter At noon the bladder was washed, at first with warm water and then with a solution of boracic acid Then, during the experimental period, the bladder was emptied at the end of each hour, without washing it When the last portion of urine was removed, the bladder was washed with boracic acid solution to prevent infection The results are summarized in the following table and are graphically presented in the accompanying chart

Influence of dextrose solutions upon the hourly secretion of urine

TIME	FASTING 2 DAYS	FASTING 3 DAYS 150 cc WATER	STANDARD DIET 18 HOURS BEFORE FOOD				
			150 cc water and				200 cc water and 75 grams dextrose
			20 grams dextrose	50 grams dextrose	50 grams dextrose	50 grams dextrose	
p m							
12 00–1 00	7 0	28 0	11 5	4 5	7 0	4 0	6 5
1 00–2 00	2 0	27 0	23 0	6 0	7 5	4 0	6 0
2 00–3 00	4 5	28 0	13 0	8 5	12 0	12 5	7 0
3 00–4 00	2 5	17 5		22 0	100 0	88 5	18 5
4 00–5 00			28 5		66 5	19 0	89 0
5 00–6 00					22 0	10 0	
6 00–7 00					16 0		
Total	18 0	100 5		41 0	231 0	138 0	127 0

It is apparent from these figures that, if water be given alone, it is quickly eliminated If the same quantity of water be given

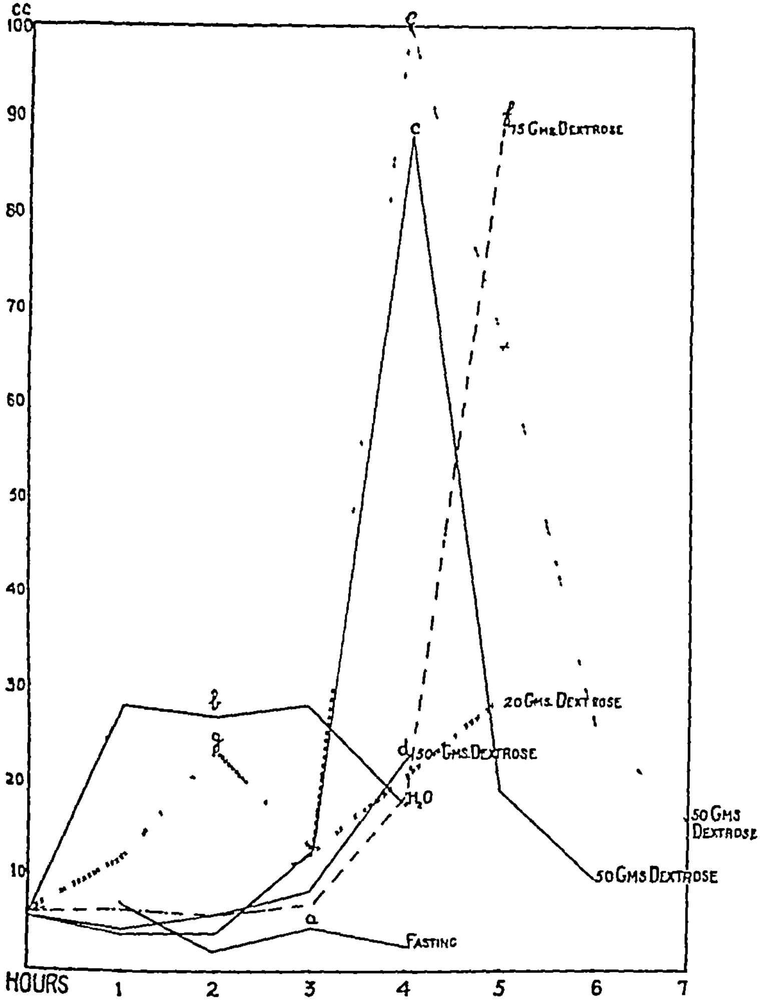

CHART ILLUSTRATING THE INFLUENCE OF INGESTED DEXTROSE SOLUTIONS UPON THE HOURLY VOLUME OF THE URINE

Straight lines = hourly urinary secretion during (*a*) fasting, (*b*) after 150 cc water, (*c*) 50 grams dextrose in 150 cc water, (*d*) 50 grams dextrose in 150 cc Dotted line (*e*) = 50 grams dextrose in 150 cc water Dash and dot line (*f*) = 75 grams dextrose in 200 cc Star line (*g*) = 20 grams dextrose in 150 cc

with 20 grams of dextrose, there is at first some hindrance to its elimination If 50 grams of dextrose be added to 150 cc of water, the result of its ingestion is to reduce the quantity of urine eliminated during the first two hours to practically the fasting minimum, during the third hour there is a slight increase and during the fourth hour the elimination of urine rises to large volume, declines in the fifth hour and becomes greatly reduced in the sixth and seventh hours A similar picture is presented when 75 grams of dextrose are given in 200 cc of water, only the minimal excretion lasts three hours instead of two, there is a slight increase during the fourth hour and the large increase in volume takes place in the fifth hour In Dog II in the previous paper, after giving 50 grams of dextrose, the fourth hour marked the end of the high metabolism and it is here found to be the hour of large urinary excretion In the same dog, after giving 75 grams of dextrose, the high metabolism continued during the fifth hour, which corresponds to the hour of the largely increased output of urine in the present experiment

The *nitrogen elimination* is shown in Table II These results, however, are not as reliable as those given for Dog II in the last paper because the bladder was not washed at the end of each period, and strictly comparable results could not, therefore, be achieved In general, however, a confirmation of the former results has been obtained, there is a small output of nitrogen during the period of diminished urine secretion and a larger output during the period of diuresis

e The hemoglobin content of the blood

Making use of the same dog, blood for hemoglobin tests was obtained every hour immediately after catheterization The blood was obtained by cutting a small ear vessel, usually an artery, and taking a sample of the freely flowing blood The incision was reopened every hour with little difficulty and the ear was washed with an antiseptic solution after taking the blood The determinations were made by the Fleischl-Miescher method, using accurate pipette dilutions A count of the red blood cells was made as a check with regard to the reliability of the hemoglobin determinations It was repeatedly found that twenty-four

TABLE II

The effect of ingested dextrose solutions upon the quantity of urine eliminated and upon the dilution of the blood as indicated by the per cent of hemoglobin in it

DIET	TIME	URINE PER HOUR	NITROGEN PER HOUR	HEMOGLOBIN AT END OF HOUR
	p m	cc	gram	per cent
Starved 48 hours	12 00-1 00	7 0		104
	1 00-2 00	2 0		100
	2 00-3 00	4 5		99
	3 00-4 00	2 5		102
150 cc H_2O at 12 00	12 00-1 00	28 0		103
	1 00-2 00	27 0		101
	2 00-3 00	28 0		101
	3 00-4 00	17 5		103
20 grams dextrose, 150 cc H O at 12 00	12 00-1 00	11 5		99
	1 00-2 00	23 0		100
	2 00-3 00	13 0		95
	3 00-4 00	Lost		95
	4 00-5 00	28 5		96
50 grams dextrose, 150 cc H_2O at 12 00	12 00-1 00	8 0		92
	1 00-2 00	6 5		83
	2 00-3 00	4 5		81
	3 00-4 00	9 0		97
	4 00-5 00	Lost		
50 grams dextrose, 150 cc H O at 12 00	12 00-1 00	4 5	0 0730	102
	1 00-2 00	6 0	0 1039	95
	2 00-3 00	8 5	0 2162(?)	
	3 00-4 00	22 0	0 0969	90
	4 00-5 00			100
50 grams dextrose, 150 cc H O at 12 00	12 00-1 00	7 0	0 1418	108
	1 00-2 00	7 5	0 1769	99
	2 00-3 00	12 0	0 1601	98
	3 00-4 00	100 0	0 1797	98
	4 00-5 00	66 5	0 1516	102
	5 00-6 00	22 0	0 1336	107
	6 00-7 00	16 0	0 1095	106
50 grams dextrose, 150 cc H_2O at 12 00 noon	12 00-1 00	4 0	0 0679	109
	1 00-2 00	4 0	0 0927	80
	2 00-3 00	12 5	0 1683	85
	3 00-4 00	88 5	0 1572	90
	4 00-5 00	19 0	0 1292	105
	5 00-6 00	10 0	0 1404	110
75 grams dextrose, 200 cc H_2O at 12 00	-12 00			102
	12 00 -1 00	6 5	0 0927	100
	1 00 -2 00	6 0	0 0997	100
	2 00 -3 00	7 0	0 1404	101
	3 00 -4 00	18 5	0 1263	
	4 00 -5 00	89 0	0 1544	

hours after food, the dog showed 100 per cent of hemoglobin in the blood or slightly over 100 The method was sufficiently reliable to demonstrate that in fasting and after giving 150 cc of water, the hemoglobin remained constant in quantity, that, after giving 20 grams of glucose in 150 cc of water, there was a slight fall in the amount of hemoglobin, and that giving 50 grams of dextrose in 150 cc caused little or no effect during the first hour but during the second hour it may frequently bring about a fall of 10 to 20 per cent in the percentage of hemoglobin present in the blood A rise to its former value takes place only after the elimination of water from the blood by the kidney These experiments show that after giving dextrose in solution *per os*, a hydremic plethora may be established in the presence of a greatly diminished secretion of urine, being a direct contradiction of the theories of Starling already discussed, according to which the excretion of urine is greatly favored by a condition of hydremic plethora

DISCUSSION AND SUMMARY

After the ingestion of 50 grams of dextrose in 150 cc of water, there is a rapid absorption of dextrose during the first hour, the sugar in the blood rises above its normal content and the hemoglobin content is not profoundly changed (the absorption of water is probably slight owing to the osmotic power of the dextrose solution in the stomach) At the end of the second hour, between two-thirds and three-quarters of the sugar ingested has been absorbed, relatively little has been retained by the liver as glycogen, the sugar percentage has become normal and the blood usually more dilute as shown by a fall in the percentage amount of hemoglobin The dilution has taken place as a result of the increased osmotic power of the blood due to the increase in sugar content found at the end of the first hour and the metabolism is 20 per cent higher than before and is at the expense of dextrose, this being due to the generous distribution of freely dissolved and uncombined dextrose molecules to the tissues (see last paper) This condition lasts through the third hour During the fourth hour the absorption of dextrose is completed, the urinary secretion, the volume of which has been almost at the fasting level, suddenly increases very largely, the hydremic plethora of the blood tends to diminish and this

hour is the last hour of the increased metabolism In one instance, at the end of the fourth hour, a large quantity of glycogen was found in the liver (a condition not found at the end of the previous hours) which suggests that the liver may have been active in the removal of the increased quantity of freely diffusible dextrose previously within the blood tissues With the return of the blood to its normal volume, the percentage content of dextrose is not altered through the concentration of the blood The fifth hour, marked by the return of the metabolism to its basal value, and which may be characterized by a lowered respiratory quotient, shows a lessened quantity of urinary output and a normal or above normal hemoglobin percentage in the blood

With 20 grams of sugar these reactions are much less marked

With 75 grams of sugar the high metabolism and the diminution of the volume of urinary secretion lasts an hour longer than with 50 grams of dextrose

STUDIES IN BACTERIAL METABOLISM VII

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(Received for publication, July 17, 1912)

A most fundamental principle of bacterial metabolism may be expressed concisely by stating that "Fermentation takes precedence over putrefaction,"[1] that is to say, bacteria in general which can utilize both carbohydrate and protein, act upon the former in preference to the latter when both are present in the same medium

In view of the confusion attending the use of the terms putrefaction and fermentation, they must be sharply defined By fermentation is meant "the action of microorganisms upon carbohydrate,' and by putrefaction is meant "the action of microorganisms upon nitrogenous substances "[2]

Bacteria in common with all known living things need nitrogen to build up their bodies, it is self-evident, therefore, that even when carbohydrate is being fermented, enough protein must be broken down to satisfy their nitrogen requirements Bacterial activity, therefore, must be sharply differentiated into two distinct processes, the structural and the vegetative, both functions being essential for their metabolism

Nitrogen is indispensable for the structural process, hence bacteria must have nitrogen in their dietary With the vast majority of bacteria, however, the vegetative process may be satisfied either by utilizable carbohydrate or by protein Whenever bacteria can utilize both carbohydrate and protein for their vegetative activity (for fuel) and both are present in the medium in which these

[1] Kendall *Journ Amer Med Assoc* , lvi, pp 1084–1088

[2] For a more complete discussion of these terms, see Kendall *Journ of Med Res* , pp 140-144, 1911

organisms are growing, the carbohydrate is invariably selected in preference to the protein Even when protein is being utilized for vegetative purposes, the bacteria actually eliminate nitrogen from the protein molecule and apparently utilize only the carbon, hydrogen and oxygen for their energy metabolism (fuel) This amounts practically to the use of carbohydrate in the last analysis for fuel purposes

In the past, but little effort has been made to study bacterial metabolism quantitatively, at least from the comparative standpoint, yet it is largely from the comparative study of different types of bacterial metabolism that the fundamental principles can be elucidated This lack of diligence cannot be explained wholly by the inadequateness of the older methods, it is rather attributable to rigid adherence to the narrow, botanical idea of morphology and differentiation of bacteria instead of the broader, dynamical consideration of bacterial activity The few painstaking studies which have been made along these lines have failed for the most part because of the neglect of the carbohydrate factor in the media in which such experiments have been carried out For these reasons, it is impossible to quote from the literature any studies which are carried out along lines similar to those presented below

The results upon which this summary is based have been derived from the comparative study of a considerable number of bacterial types using methods of far greater accuracy than those previously available The types of bacteria represented in this series cover those commonly met with in bacteriology, and a sufficiently large number of strains of each type have been examined to give definite assurance of the physiological and chemical limits of each species The organisms selected for this work have been drawn largely from the normal and pathological flora of the gastro-intestinal tract where dietary alternations, comparable with those to which these bacteria are subjected culturally, are commonly met with

The methods employed have been described critically in previous communications,[3] and will not be referred to here other than to state that the limits of error are much less than the thickness of the lines shown in the various curves at the end of the paper

Similarly, the analytical results have been presented in previous

[3] Folin and Farmer this *Journal*, xi, p 493, Folin and Macallum *ibid*, xi, p 523, Kendall and Farmer *ibid*, xii, pp 13, 19, 1912

articles[4] It is the purpose of this communication to plot, analyze and synthesize these results The appended curves, accurately constructed from these analytical figures, show *graphically* the effects of carbohydrate upon bacterial metabolism These curves furthermore illustrate in a striking manner the diversity of types of bacterial metabolism in media of the same composition, yet in spite of these diversities the sparing action of carbohydrate for protein is apparent, except in the strictly "carnivorous" organisms, *B alcaligenes* and H-61

The frankly pathogenic organisms associated with toxemia in the human body, as typhoid and dysentery (both Shiga and Flexner), break down but little protein, as is shown by the small amount of ammonia liberated in the sugar-free medium, when they are using protein for fuel as well as for structural purposes The amount of ammonia liberated by the less frankly pathogenic organisms increases progressively as the more saprophytic types, *e g*, *B proteus*, are approached At first sight, cholera might seem to be an exception to this generalization It must be remembered, however, that cholera may be a rapidly fatal disease, the entire course from infection to death taking place within twelve hours This contrasts strikingly with diseases such as typhoid, where the incubation period alone is about fourteen days on the average The proteolytic activity of cholera may be effectively checked by the presence of dextrose

It will be seen from the curves that the proteolytic activity, but not the structural activity of bacteria (except the strictly carnivorous types), can be arrested by the presence of utilizable carbohydrate The products of proteolytic activity, which are only formed when bacteria are utilizing protein for fuel, are alkaline, nitrogenous substances, the products of fermentation, on the contrary, which are formed when bacteria are utilizing carbohydrate for fuel, are non-nitrogenous, acid products It must be remembered that all known true toxins are nitrogenous, while acids produced by fermentation are at best but irritants and are for the most part non-nitrogenous It would appear, therefore, that the production of toxic substances of bacterial origin must be the result of proteolytic (putrefactive) activity rather than of fermentative activity

[4] Kendall and Farmer this *Journal*, xii, pp 13, 19, 215, 219, 465, 469, 1912

The importance of the sparing action of carbohydrate for protein in the light of toxin production must be apparent

We believe that the principle elucidated above is not limited to bacteria alone, but that it is in reality a general and fundamental principle of cellular metabolism

Explanation of Charts

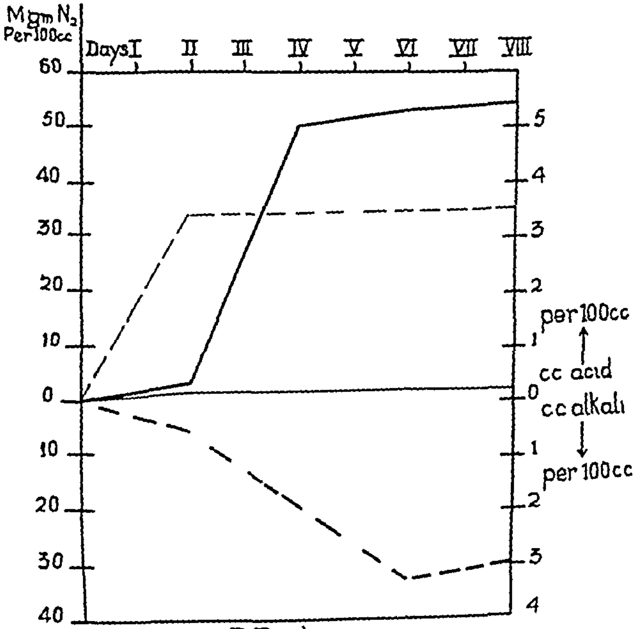
The solid, heavy line represents the production of ammonia in milligrams of nitrogen per 100 cc of culture medium in sugar-free broth

The solid, light line represents the production of ammonia in milligrams of nitrogen per 100 cc of culture medium in dextrose broth

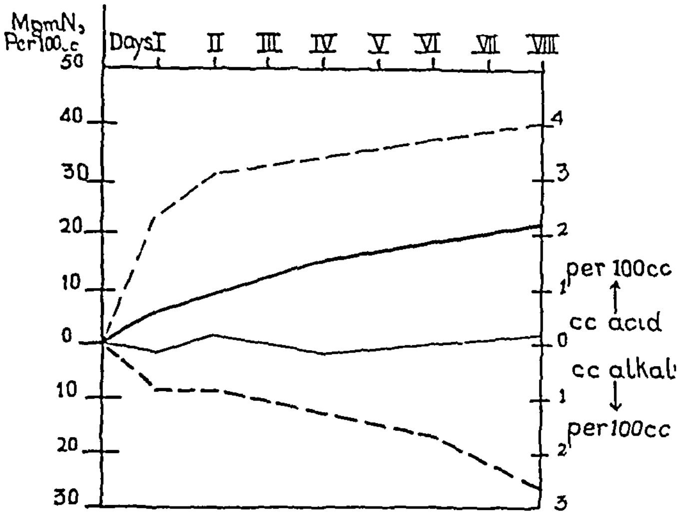
The broken heavy line represents reaction in terms of cubic centimeters of normal acid or alkali per 100 cc of sugar-free culture medium

The broken light line represents reaction in terms of cubic centimeters of normal acid or alkali per 100 cc of dextrose broth

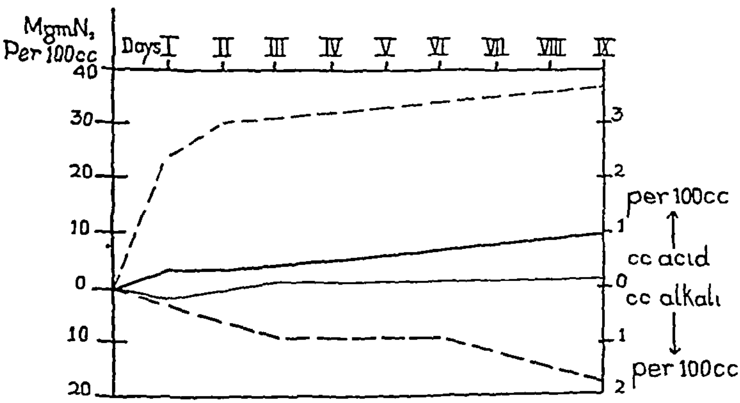
The composite curve illustrates graphically the relative amounts of ammonia produced by various types of pathogenic and saprophytic bacteria, for convenience the different types are drawn in the same figure to bring out forcibly the difference in ammonia production in sugar-free broth between these types It will be seen that the production of ammonia in dextrose broth is essentially the same for all these organisms, excepting those which can utilize no sugar This ammonia production in sugar-containing broth is a measure of the nitrogen needs of bacteria for structural purposes as contrasted with the fuel needs



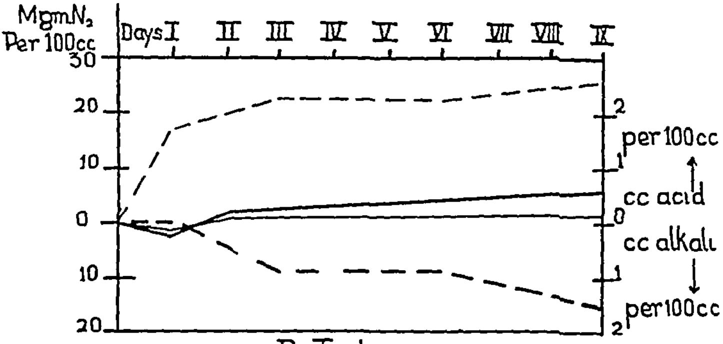
B Proteus



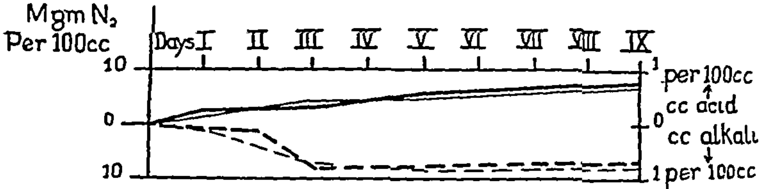
B Coli



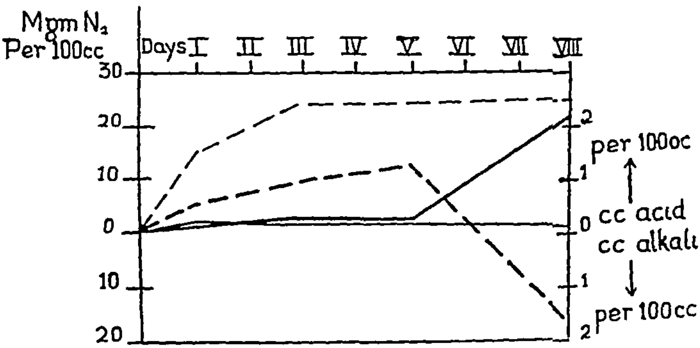
B Paratyphosus β



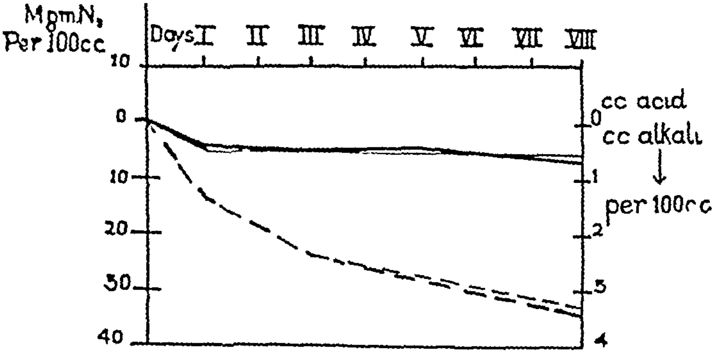
B Typhosus



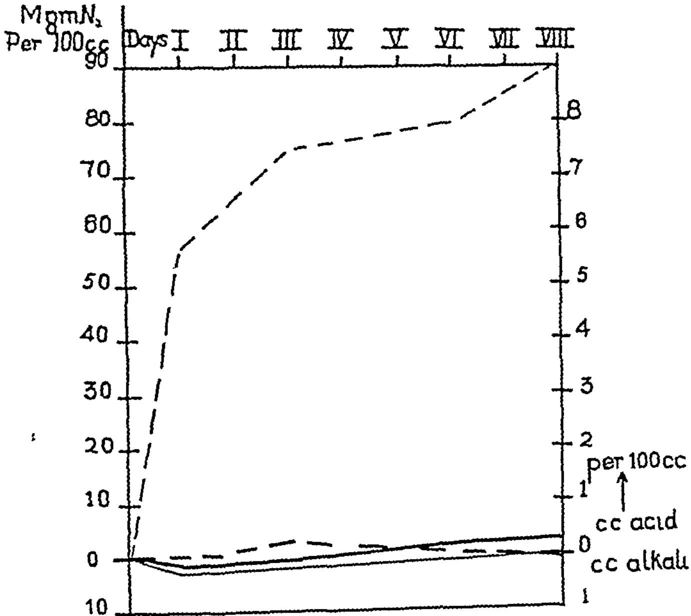
B Alcaligenes



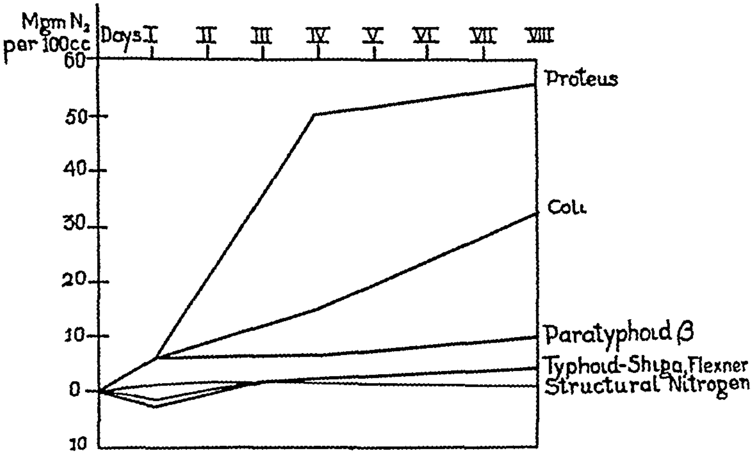
Mic Aureus



Vibrio H-61



Streptococcus 34



Composite Curve

THE BEHAVIOR OF FAT-SOLUBLE DYES AND STAINED FAT IN THE ANIMAL ORGANISM[1]

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(Received for publication, August 26, 1912)

INTRODUCTION

Since Daddi[2] discovered that Sudan III, when fed incorporated with fat, is absorbed and laid down in the adipose tissue of animals, various experimenters have used the dye as a means of studying problems connected with fat metabolism The possibilities of this method have not been exhausted, and the present investigation was aimed to extend the application of fat-soluble dyes to the solution of some of the unanswered questions

The dyes used were Sudan III (Kahlbaum), Biebrich Scarlet (Aniline Red, R Medicinal Merck), Indophenol (H A Metz and Company), Oil Soluble Green (H A Metz and Company), Oil

[1] A preliminary report of some of the data recorded here was presented to the Society for Experimental Medicine and Biology (cf *Proceedings*, viii, p 126, 1911) The essential facts in this paper are taken from the dissertation presented by Amy L Daniels for the degree of Doctor of Philosophy, Yale University, 1912

[2] *Arch ital de biol* , xxvi, 142, 1896

Orange (National Aniline and Chemical Company), Blue Base (Hudson River Aniline Color Works), Dandelion Brand Butter Color (Wells, Richardson and Company), and Annatto These are water-insoluble compounds which are soluble in fat, fatty acids, alcohol, ether, chloroform, benzene and bile, as well as in solutions of the isolated bile salts They were introduced, dissolved in fat or in lecithin emulsions of oil,[3] either by feeding or by intravenous, subcutaneous or intraperitoneal injections The dyes deposited in the fatty tissue and secreted milk of the experimental animals were easily detected by the color, those in the glandular, muscular, and nervous tissue, and in the fluids of the body—the blood, lymph and bile—were less easily determined In all cases 2-gram portions of the tissue to be examined were minced, dried with anhydrous sodium sulphate and extracted with ether The ether extracts were filtered, allowed to evaporate in white porcelain dishes, and the colors of the residues were noted The blood and lymph were also dried with anhydrous sodium sulphate, the bile was similarly extracted with ether after being dried down with calcium oxide to form ether-insoluble compounds of the bile pigments

DEPOSITION OF FAT-SOLUBLE DYES IN ANIMAL TISSUES

With the exception of the meal worm, *Tenebrio molitor*,[4] the infusoria (Staniewicz) and possibly the cow[5] the adipose tissue has been found to be stained in those animals into which fat, stained with Sudan III, has been introduced The animals investigated, the methods of introducing the stain and the results obtained, are summarized in the table on pages 74 and 75

Although the time required to stain the adipose tissue of animals of different species has been noted only incidentally, it would seem from the results reported that it varies considerably Riddle has observed that rabbits and turtles absorb Sudan III less rapidly than the fowl, in which the fatty tissue is colored after one or two days' feeding The red stain appeared in the yolk of the eggs of hens,

[3] This emulsion, supplied by Fairchild Bros and Foster, consisted of 5 per cent lecithin, 45 per cent peanut oil and 50 per cent water

[4] Biedermann *Arch f d ges Physiol*, lxxii, p 105, 1898

[5] S H and S P Gage *Science*, xxviii, p 494, 1908, *Anatomical Record*, iii, 1909

and in the milk of rats after one feeding, whereas the cow observed by S P and S H Gage[6] gave no evidence of Sudan absorption after four days of Sudan feeding

EXPERIMENTAL Feeding experiments with *Sudan III* were carried out with rats, cats, guinea pigs, pigeons, hens, frogs, a cow and a goat The results were comparable with those of the earlier investigators After a single feeding of deeply stained food, colored fat was found in the milk of cats and rats, and in the egg of the hen Pigeons, after five days, showed a distinct pink coloration of the subcutaneous tissue through the skin, at autopsy, the fatty tissue was found to be distinctly stained Three guinea pigs, to which were given two gelatin capsules, each containing 80 mgms of Sudan III, every second day for four weeks, gave no evidence of stained tissue, two guinea pigs, given 2 cc of stained oil every second day for three weeks, contained faintly pink adipose tissue Frogs were fed for three weeks during the hibernating period with meat liberally mixed with stained oil, throughout the experiment they were kept in a room at 20°C In no case did the fatty tissue of these become stained The cow secreted no stained milk even after seven successive feedings of 7 5 grams of Sudan dissolved in oil, whereas the milk of the goat was faintly, but distinctly, pink after one feeding of Sudan stained food

It will be observed that, in general, those animals (rats, cats and fowls) which absorb fat readily, give evidence of Sudan-stained fat in less time than those, like the guinea pig and cow, in which fat forms a smaller factor in the diet

Biebrich Scarlet, which resembles Sudan III in its solubilities, and is not affected by dilute solutions of acids and alkalies, was fed to pigeons, rats and cats, with results comparable with those obtained with Sudan III The subcutaneous tissue was colored pink

Feeding experiments with *indophenol-blue* were unsuccessful This dye, unaffected by dilute alkalies, changes to pale yellowish green when treated with dilute hydrochloric acid This color change in the stomach made it impossible to detect the dye absorbed In rabbits and pigeons after subcutaneous injections of oil emulsions colored with the blue dye no fatty tissue was found

[6] *Anatomical Record,* iii, 1909

Animals stained with fat-soluble dyes

INVESTIGATOR	ANIMAL	DYE	METHOD OF INTRODUCING DYE	TISSUES AFFECTED	REMARKS
Daddi ('96)	Rabbits	Sudan III	Feeding stained oil	Subcutaneous	
	Guinea Pigs	Sudan III	Feeding stained oil	Subcutaneous	
	Pigeons	Sudan III	Feeding stained oil	Subcutaneous	
Biedermann ('98)	*Tenebrio molitor*	Sudan III	Feeding stained oil	None	Body fat uncolored
		Alkanna	Feeding stained oil	None	Body fat uncolored
Sitowski ('05)	Caterpillar	Sudan III	Feeding stained wool	Body and Eggs	
Hofbauer ('05)	Guinea Pigs	Sudan III	Feeding stained fat	Adipose tissue	
Riddle ('08)	Fowl	Sudan III	Stained food	Adipose tissue and eggs	
Riddle ('10)	Rabbits	Sudan III	Stained food	Adipose tissue	Rabbits become stained less readily than fowl
	Turtles	Sudan III	Stained food	Eggs	Turtles become stained less readily than fowl
	Rabbits	Sudan III	Parenteral injection	Adipose tissue	
			Intravenous injection	Adipose tissue	
S P and S H Gage ('08)	Fowl	Sudan III	Stained food	Adipose tissue and eggs	Adipose tissue of chicks from stained egg is colored

S P and S H Gage ('09)	Rats	Sudan III	Stained food	Adipose tissue	Milk colored, young born not colored
	Guinea Pig	Sudan III	On carrots	No examination	Young not colored
	Cow	Sudan III	Stained food	No examination	Milk not colored
Staniewicz ('10)	Infusoria	Sudan III	Feeding stained fat	None	Body fat uncolored
Mann*	Cats	Scharlach Rot	Feeding stained oil	Adipose tissue	
Mann*	Cats	Alkanna	Feeding stained oil	Adipose tissue	

*These data were sent to the writer by Prof Gustav Mann and it is with his permission that they are given here

stained The blood of rabbits taken from two to six hours after intravenous injections of indophenol-blue dissolved in oil emulsion yielded pink residues on extraction

These results point to the reduction of the indophenol-blue to indophenol by the tissues The presence of active reductases in the various tissues of the animal body has been observed by Ehrlich,[7] Herter,[8] Harris[9] and others Heffter[10] reports that the liver is particularly rich in this enzyme, a fact which was further demonstrated by us as follows

Ground liver tissue, to which oil stained with indophenol-blue had been added, was allowed to autolyze in the presence of toluene at a temperature of 30°C After twenty-four hours, the mixture had lost its blue color and had become pink, the addition of hydrogen peroxide brought back the blue color No change in color took place in a control experiment, carried out with boiled liver tissue under identical conditions

The localization of fat-soluble dyes in the tissues

Analysis of the various tissues of the animal body shows that the largest quantity of fat (ether extract) is found in the subcutaneous tissue, the fatty tissue of the abdominal cavity and the bone marrow, however, the muscular, glandular and nervous tissues contain estimable amounts It is reasonable to suppose, therefore, that animals containing Sudan-stained adipose tissue would likewise have stained fat in the other fat-bearing tissues, especially since this dye readily reveals the presence of fat in histological sections of these tissues The only investigators who even suggest that the fat of other than the adipose tissues may not be colored are Mann[11] and S P and S H Gage[12] The basis for Mann's statement that "animals fed on oil colored with Sudan III show only the adipose tissue stained" is not clear S P and S H Gage failed to find the stain in the nerve fibres of the chicks developed from the Sudan-stained eggs, although the adipose tissues of these were distinctly colored

[7] *Das Sauerstoffbedürfniss des Organismus*, Berlin, 1885

[8] *Amer Journ of Physiol*, xii, pp 207, 457, 1904–5

[9] *Bio-chem Journ*, v, p 143, 1911

[10] *Medizinisch naturwissenschaftliches Archiv*, i, p 81, 1907–8

[11] *Physiological Histology*, 1902, pp 36–7

[12] *Science*, xxviii, p 494, 1908

Bondi and Neumann[11] found that the bone marrow and livers of rabbits were distinctly blue after the injection of an emulsion of fat, stained with indophenol, and that the Kupfer cells of the livers of rabbits became distinctly pink after the injection into the circulation of an oil emulsion stained with Scharlach Rot The animals were killed a few hours after the injection, the adipose tissue had not become stained in this short time, and the fact that the liver cells contained the color of the dye injected cannot be taken as proof that these cells store fat The results of subsequent experiments in this investigation pertaining to the mode of elimination of fat-soluble dyes, to which reference will be made later, have thrown some light upon this point, and make it evident that these observations of Bondi and Neumann may be otherwise interpreted

Experimental In order to ascertain whether stained fat, other than that in the distinctly adipose tissue, is present in the bodies of animals into which fat-soluble dyes have been introduced, 2-gram portions of the tissues to be examined were freed, as far as possible, from extraneous fat and connective tissue, finely divided, dried and extracted with ether in accordance with the method already described The dyes were administered dissolved in olive oil or in lecithin emulsion of peanut oil The results are summarized in the table on pages 78 and 79

Discussion Negative results were always obtained from nervous and renal tissues, from muscle when it was freed from connective tissue or extraneous fat as in starvation, and in general from liver tissue Livers however from which blood had not been removed by perfusion or bleeding sometimes showed traces of the dye In two cases the livers from rats which had been fed on a diet containing 75 per cent of deeply stained lard, yielded considerable quantities of the dye These livers were distinctly pink, owing undoubtedly to the storage of the absorbed fat in the liver cells Microscopic examinations of frozen sections, however, failed to disclose the dyes, even when chemical isolation demonstrated their presence

The explanation of these results is not clear It may be that the form of the fat in the nervous, muscular and glandular tissues

[11] *Zentralbl f Biochem u Biophysik*, x, p 1453, 1910

Tissues stained with fat soluble dyes

+ means present, − means not detected, ? means trace

ANIMAL	DYE	MODE OF INTRODUCTION	RESIDUE FROM ETHER EXTRACT OF: Brain	Kidney	Marrow	Muscle	Liver	Adipose tissue	REMARKS
Cat A	Sudan	Feeding	−			?	−	+	
Kitten	Sudan	Intra-peritoneal injection	−			−	−	+	
Cat B	Sudan	Intra-peritoneal injection	−	−	+	?	−	+	
Cat C	Sudan	Feeding	−	−	+	?	−	+	
Cat D	Sudan	Feeding	−	−	+	−	−	+	
Pigeon N	Annatto	Feeding	−		−		−	−	
Pigeon A	Sudan	Feeding	−			−	−	−	Died from starvation
Pigeon B	Sudan	Feeding	−		+	−	+	−	Died from starvation
Pigeon D	Sudan	Feeding	−		+	−	−	−	Died from starvation
Hen	Sudan	Feeding	−		+	−	−	+	Died as the result of phosphorus poisoning
Rat XI, 24	Sudan	Feeding	−		+	−	−	+	
Rat XI, 26	Sudan	Feeding	−	−	+	−	−	+	
Rat XI, 27	Sudan	Feeding	−			−	−	+	
Rabbit XI, 28	Sudan	Feeding		−		−	−	+	
Rabbit I, 5	Sudan	Feeding					−	+	
Rabbit 9,	Sudan	Feeding					−	+	
Pigeon I	Indophenol	Feeding	−				−	−	

Rat C	Sudan	Feeding	–	–		–	–	–	Starved 60 hours
Rat D	Sudan	Feeding	–	–		?	–	+	Starved 24 hours
Rat E	Sudan	Feeding		–		–	–	–	Starved 60 hours
Rat 5	Sudan	Feeding		–	+	–	+	+	Starved 18 hours
Rat XI, 12	Sudan	Feeding			+		+	+	Food contained 75 per cent stained fat
Rat XII, 9	Sudan	Feeding			+		+	+	Food contained 75 per cent stained fat
Rat XI, 5	Sudan	Feeding	–				+	+	Died as the result of phosphorus poisoning Liver dark red
Rat II,10	Biebrich Scarlet	Feeding					–	+	

of the body is quite different from that in the adipose tissue—that it is held in some loose chemical combination which is no longer capable of taking up the stain The present methods of fat extraction and staining may result in a disintegration of this complex molecule MacLean and Williams[14] have advanced the theory that the fat removed by extraction from animal tissues does not represent the form in which the fat exists in these tissues, and that the fat is made evident as the result of certain post-mortem changes by which the compound is broken up and the fat liberated Leathes[15] and Abderhalden and Brahm[16] have suggested that the fat of the active tissues differs from that of the storage tissue In the present investigation it was found that the isolated ether-soluble substances of the brain can take up the stain This observation, together with the fact that the nervous tissue of Sudan-stained animals is always free from the dye, even when the embryonic fat contained an abundance, as was demonstrated by S P and S H Gage[17] in chicks developed from the stained eggs, adds weight to the theory outlined above

An explanation of the fact that in a large number of the experiments the liver tissue was found to be free from the dye, is afforded by the observation that *the fat-soluble dyes are more soluble in bile than in fat, and when these dyes are introduced into the body in solution in the fat they are eliminated in the bile* Added evidence in favor of this explanation is found in the fact that the fat complex in the liver is not incapable of holding the dye in combination This is shown by the following experiment

A solution of Sudan III in bile was injected, under pressure, into the common bile duct of a rabbit After 20 cc had been forced in, the liver was removed, perfused with physiological saline solution, comminuted, washed in cold running water for twenty-four hours and filtered, the residue, which was distinctly pink, was washed until the filtrate gave no test for bile salts with Pettenkofer's reaction, ether extracts of the dried residue were distinctly pink There could be no doubt that the fat had absorbed the stain

CONCLUSIONS Stained fats, introduced into the animal body intraperitoneally, intravenously, subcutaneously or by absorption

[14] *Bio-chem Journ*, iv, p 455, 1909
[15] *Problems in Animal Metabolism*, 1906, p 72
[16] *Zeitschr f physiol chem*, lxv, p 330 1910
[17] *Science*, xxviii, p 494, 1908

from the alimentary tract, are laid down in the adipose tissue and marrow

The renal and nervous tissues are free from the stain, even when the fatty tissue is deeply colored, muscle tissue, when freed from fat, as in extreme starvation, contains no stained fat, the dye is found in the liver only when the blood contains an abundance, as in starvation, or when the animal has been fed food containing a large amount of stained fat a few hours before the examination

Liver fat, *in situ*, is capable of taking up the stain

Indophenol-blue is reduced in the body, this reduction takes place, in part, in the liver, hence adipose tissue is not stained with this dye

AVAILABILITY OF STAINED FAT IN METABOLISM

Riddle[18] has suggested that adipose tissue stained with Sudan III is less available to the organism than unstained adipose tissue Inasmuch as the dye enters into no chemical union with the fat, but is merely dissolved therein,[19] it does not seem probable that the Sudan III can so change the nature of the fat that it cannot be used as effectively by the organism as unstained fat An indifferent material, like Sudan III, might be toxic, or might form toxic combinations in the body, and thus affect organs dealing with fat combustion, but that the fat itself is rendered unavailable scarcely seems tenable The non-toxicity of Sudan III has been shown by feeding animals over long periods of time without apparent deleterious results

Experimental, In order to determine if Sudan-stained fat is less available to the organism, starvation experiments were carried out with Sudan-stained rats and pigeons, comparable experiments were conducted with normal animals

1 *Pigeon B* Fed with pulverized dog biscuit, lard deeply stained with Sudan III, and cracked corn for three weeks before the beginning of the fasting period Subcutaneous tissue became pink Weight of pigeon at beginning of fast was 297 grams Death in ten days It had lost 116 5 grams, 39 per cent of its initial weight, all visible fat had disappeared No pink color was to be seen A slight trace of Sudan III was found in

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[18] *Journ of Exp Zoöl*, viii, p 163, 1910

[19] Michaelis *Virchow's Archiv*, clx, p 263, 1901

ether extract of the tail gland, bone marrow and liver, the muscle, kidney and brain contained no trace of the dye

2 Pigeon C Preliminary feeding same as B Subcutaneous tissue became noticeably pink Weight at beginning of fast 294 grams Death in eleven days Loss of weight 165 grams, 56 per cent No visible fat remained, tissues showed no pink color, ether extract of liver and bone marrow slightly pink, of muscle, kidney and brain, colorless

3 Pigeon D Fed Sudan-stained food as in B and C After sixteen days of fasting this animal died It had lost 41 per cent of its initial weight All visible fat and stain had disappeared from the body The ether extract of the bone marrow was slightly pink, that from the liver and muscle showed no pink color

4 Pigeon E A normal well-fed bird, was starved for sixteen days, during which it lost 229 grams or 54 per cent of its initial weight All visible fat had disappeared from the body

5 Pigeon F A well-fed normal bird which died fifteen days after the fasting period began The loss in weight was 144 grams or 45 per cent of its initial weight All visible fat had disappeared

It should be noted that Pigeons B and C were kept during November in an unheated room with the windows open This doubtless explains their earlier death as compared with pigeons D, E and F which were kept at about 20°C In every case, however, the fatty tissue had entirely disappeared from the body

Experiments with rats gave similar results

6 Rat A Fed with ground dog biscuit mixed with lard deeply stained with Sudan III for seven days Fasting period, three days Loss in weight, 42 grams, 42 per cent of initial weight The body was free from all traces of visible fat and stain

7 Rat C Preliminary feeding period, same as A Fasting period approximately sixty hours All visible fat disappeared from the body The ether extract of the brain, liver, muscle, kidney and subcutaneous tissue left no pink residue

8 Rat B A normal well-fed rat, which died after a 60 hours' fast The loss in weight was 40 grams or 22 per cent of its initial weight The body was free from all visible fat

9 Control experiment Rat D Was fed on Sudan-stained food as in the previous experiments, for seven days The subcutaneous tissue, omentum and fatty tissue about the kidneys were deeply pink

The result of the control experiment affords evidence that the adipose tissue of the experimental animals was similarly stained at the beginning of the fasting periods The further observation was made that rats, and in some cases rabbits, stained as the result of feeding with deeply stained fat-rich food, excreted urines which

were distinctly pink, such urines were found to contain both fat and Sudan III

In two experiments Sudan-stained pigeons were fed with unstained foods after long fasting periods—other pigeons, fasting the same length of time and under similar conditions, had died At autopsy, the fatty tissue of these was found to be unstained

10 Pigeon G Fed with Sudan-stained fat, described in protocols 1–3, starved thirteen days, loss of weight, 104 grams or 29 per cent It was re-fed and examined some months later All trace of Sudan had disappeared, the ether extract of the tissues left no pink residue

11 Pigeon A Previously fed with Sudan-stained food, starved eleven days, loss of weight, 104 grams or 32 per cent It was re-fed until it had gained 16 grams Upon examination, no stained tissue was found Ether extract of subcutaneous fat, tail gland and omentum showed no pink color

Discussion The results of these trials are not in agreement with those reported by Riddle Sudan-stained pigeons and rats died in no less time than the unstained control animals In both cases the visible fat had entirely disappeared, and, in the stained animals, the dye as well Those animals which were fed after long fasting periods until there was a marked increase in body weight, contained no trace of the former Sudan-stained fatty tissue One must conclude from these results that stained adipose tissue is no less available to the organism than the non-Sudan-stained fat and that it is used quite as readily and completely

The disparity between our results and those of Riddle is difficult to explain His observations that chicks fed on stained food developed more slowly than normal chicks and that hens ceased to lay after considerable quantities of the dye had been ingested may have resulted from other causes than the ingestion of the dye It is conceivable that the apparent failure of starving stained animals in his experiments to utilize their fatty tissues as do normal animals was the result of impurities in the dye fed Mann[20] has observed that Scharlach Rot given to half grown kittens in large doses causes vomiting We gave large doses of Sudan III, put up by an American manufacturer, to two cats These died within a comparatively short time apparently from the effect of some impurity in the dye Other cats, given equally large doses of the Kahlbaum dye, experienced no ill effects Riddle's deductions from

[20] Personal communication

his second series of fasting experiments that stained animals underwent a greater percentage loss of weight during starvation than do unstained are unconvincing by reason of the fact that an important part of his weighing records was lost

THE FATE OF FAT-SOLUBLE DYES IN THE ORGANISM

The observations cited above have shown that Sudan III, deposited in the tissues as the result of adding the dye to the food, disappears completely during starvation Experiments upon cats and rats gave no reasons for thinking that this disappearance is due to elimination of the dye by the kidneys The fact that the excreta of starving Sudan-stained pigeons contained the dye and the observation that the dye was present in the gall bladders of Sudan-stained animals subjected to starvation or poisoning with phosphorus or phlorhizin turned our attention first to the elimination of fat-soluble dyes by way of the bile It is well-known that the bile is the normal path of elimination of many substances From the work of Abel and Rountree[21] on phenoltetrachlorphthalein the assumption seems justifiable that substances which leave the body exclusively by way of the bile must be insoluble in water and soluble in bile or substances contained therein

Two preliminary experiments were made upon cats, previously fed with Sudan III and starved for four days preceding the experiment The bile, collected as it was secreted by the liver, and the blood yielded pink ether extracts, while those obtained from the liver tissue, washed free of blood and bile, were colorless These results pointed to a transport of Sudan-stained fat to the liver with subsequent storage of the fat in the liver and elimination of Sudan III in the bile

The elimination of fat-soluble dyes under normal conditions

The dyes, dissolved in lecithin-oil-emulsion, were introduced into the circulations of cats, dogs and rabbits by injections into the femoral veins In each case the urine contained in the bladders, as well as the liver tissue and bile, was examined for the injected stain The approximate time of the appearance of the dye in the

[21] *Journ of Pharmacol and Exp Therapeutics*, 1, p 231, 1909

bile after its introduction into the blood stream was incidentally noted

The results of the experiments are summarized in the following table

The excretion of fat-soluble dyes introduced dissolved in fat

ANIMAL	DYE	COLOR OF DYE	RESIDUE FROM ETHER EXTRACT OF BILE	TIME OF APPEARANCE OF DYE IN BILE	DYE IN URINE	DYE IN LIVER
				minutes		
Cat II, 8	Indophenol	Blue	Blue	30†		None
Dog II, 14	Sudan	Red	Pink	30	None	Present
Dog II, 16	Sudan III	Red	Pink	20	None	None
Cat II, 21	Indophenol	Blue	Blue		None	Present*
Dog II, 21	Sudan III	Red	Pink	60†		
Cat II, 22	Oil Green	Green	Green	30	None	None†
Cat II, 23	Oil Green	Green	Green	90	None	None
Rabbit II, 28	Biebrich Scarlet	Red	Pink	60	None	Present
Dog III, 21	Sudan III	Red	Pink	60	None	None
Cat III, 24	Sudan III	Red	Pink	75	None	None
Cat IV, 21	Butter Color	Yellow	Pink	55	None	None
Cat V, 2	Oil Orange	Orange	Yellow-red	90	None	None
Cat V, 6	Blue Base	Blue	Pink	50	None	Present‡
Cat V, 15	Butter Color	Yellow	Pink		None	

* The animal died fifteen minutes after the second injection of dye

† The animal died one-half hour after the injection

‡ The addition of dilute hydrochloric acid to the liver tissue resulted in a blue color The animal died four hours after the injection

In a number of the experiments the residues from the ether extracts of the excreted bile were examined for fat The ethereal filtrates were allowed to evaporate from watch glasses, the residues were heated gently, no melting of the material took place and no grease spot was formed on soft tissue paper by this residue The dyes were excreted dissolved in bile and not in combination with fat

In some cases, the color of the residues from the ether extracts of the bile was not precisely like that of the dyes injected This change in color is the result of the passage of the dyes through the body where they are brought in contact with hydroxylions The action of dilute alkalies on the dyes outside the body causes a similar change

The residues from the ether extracts of the liver tissue were not always colored, when the animals were killed some time after the injection of the stain, or when only a small amount had been introduced, the liver was found to be free from the dye The urines were consistently free from stain

It is obvious from these results that *fat-soluble dyes, when introduced into the circulation in solution in fat, become separated from the fat and are eliminated in the bile*

Absorption of fat-soluble dyes into the portal circulation

The next experiments were designed to determine the rôles played by the bile and by the fat in the absorption of fat-soluble dyes from the intestine

22 Dog 25 kgm Fed at 7 20 a m Two hours later the animal was anaesthetized, and a cannula was inserted in the thoracic duct Twenty cubic centimeters of Sudan-stained oil were injected into the duodenum at 11 15 a m , followed by 1 gram of desiccated ox bile in solution At 12 15 the lymph was intensely pink At 2 00 p m , a cannula was inserted in the bile duct The animal was killed by bleeding at 5 30 p m Two-gram portions of the liver tissue, 10 cc samples of the blood, 50 cc of the lymph and from 2 to 4 cc of the bile were examined The ether residues from the dried lymph and bile were distinctly pink, while those from the blood and liver showed no trace of the dye

23 Cat, full-grown Fed at 8 15 a m , was anaesthetized at 9 30 a m A cannula was inserted in the thoracic duct at 10 45 a m , after 10 cc of Sudan-stained emulsified oil had been injected into the duodenum, a cannula was placed in the common bile duct and the bile collected therefrom The lymph flowed freely, and at 2 30 p m it was distinctly pink in color The ether residues from the lymph and bile were distinctly pink The blood (10 cc) taken at 5 00 p m yielded no pink residue The liver was also free from the dye

These experiments show clearly that although Sudan III, introduced with fat into the intestine, is absorbed by the lacteals and appears in the thoracic lymph, it is still absorbed and eliminated in the bile, under conditions which preclude the entrance of the lymph into the blood In the latter case, neither the blood of the general circulation nor the liver tissue is stained This behavior is explained in part by the observation that the dyes studied are more soluble in bile than in fat and by the results of the following experiments which show that the dyes may be absorbed from the intestine into the portal circulation in solution in bile

Fasting animals were anaesthetized, a cannula inserted into the bile duct and bile solutions of the various dyes used in the earlier experiments were injected into the small intestine The results are summarized below

The excretion of fat-soluble dyes absorbed from alimentary tract, dissolved in bile

ANIMAL	DYE	COLOR OF DYE	RESIDUE FROM ETHER EXTRACT OF BILE	DYE IN LIVER	DYE IN BLOOD	DYE IN URINE
Cat II, 8	Sudan III	Red	Pink	None	None	None
Cat II, 8	Blue Base	Blue	Blue	None	None	
Cat III, 10	Indophenol	Blue	Blue	None	None	None
Cat III, 10	Oil Green	Green	Brown pink	None	None	None
Rabbit III, 20	Biebrich-Scarlet	Red	Pink		None	None
Cat V, 29	Oil Yellow	Orange	Yellow pink	None		

The presence of the dye in the bile in these experiments and its absence from the blood of the general circulation show clearly that it is absorbed with the bile by the portal circulation and eliminated with the bile by the liver That none of the dye entered into the general circulation is evidenced by the fact that the blood of the animals examined—five out of six—gave no indication of even traces of the dye when tested by a method capable of detecting 0 00001 gram of Sudan III in 10 cc

The two following experiments show that when bile is not present with the dye in the intestine, no absorption of the dye in the portal circulation occurs

Stained fat was introduced into a loop of the upper intestine after this had been washed out with physiological saline solution to remove all traces of the adherent bile The bile excreted under these conditions was free from the stain, although in one case (cf protocol 24) the thoracic lymph showed that a slight amount of fat absorption had taken place, in the other experiment (cf protocol 25) both bile and lymph contained no dye until after the introduction of a bile solution into the intestinal loop, when the excreted bile was found to contain Sudan III, although the lymph was still colorless

24 Dog 20 kgm Narcotized with morphine and ether A temporary lymph cannula was inserted at 10 00 a m, and a bile cannula at 10 45 a m A 12-inch loop of the intestine was tied off just below the pylorus and washed out with physiological saline solution, at body temperature, until the washings were clear Sudan-stained oil, together with a solution of 0 1 per cent HCl, introduced to increase the pancreatic secretion, were injected into the intestinal loop at 11 00 a m Bile, collected at 12 00 m, 2 00 p m and 3 30 p m, when dried and extracted, left no pink residues Seventy cc of lymph, collected between 2 00 and 3 30 p m, contained a small amount of Sudan III, the ether extract of dried blood was unstained

25 Dog 8 1 gm Anaesthetized with morphine and ether at 9 45 a m The insertion of the temporary lymph cannula immediately preceded that of the bile cannula The intestine was ligated just below the pylorus and 14 inches below it This loop was washed out with physiological saline solution until the washings were clear Approximately 10 cc of Sudan-stained emulsified oil, together with 10 cc of 0 1 per cent HCl were introduced into this loop The bile collected at 3 30 p m left no pink residue, the lymph also was free from dye At 3 30 p m, 10 cc of a solution of desiccated ox bile were injected into the intestinal loop The bile collected at 7 30 p m, 3 5 cc, showed the presence of the dye, while the lymph taken at this time, 25 cc, left no pink color when extracted

The elimination of the dye in the bile during fat absorption, under conditions where the stained fat was prevented from entering the general circulation, was undoubtedly due to the migration of the dye from the fat to the bile in the intestine and its subsequent absorption There is no reason to believe that the dye in the excreted bile was the result of absorption of stained fat into the portal circulation Had such been the case, the dye would have been present in the excreted bile in experiment 24, as well as in the lymph

The time required for the absorption and deposition of fat, studied by means of fat-soluble dyes

The fact that fat-soluble dyes are eliminated in the bile explains some hitherto inexplicable phenomena observed in the work with Sudan III Earlier in this investigation an attempt was made to determine the length of time required to lay down the fat absorbed from the alimentary tract Stained fat was fed to rabbits and cats, and samples of blood, taken from the ear veins of the rabbits and the jugular veins of the cats, were examined for the circulating dye The stain was still found to be present in the blood of rabbits

one week after the last stained feeding, and the blood of the cats, tested from four to five weeks after the last Sudan-feeding, left distinctly pink residues Oil emulsions stained with Sudan III and Biebrich Scarlet gave similar results when injected into the circulation of rabbits The blood of these was found to contain the dye three weeks after the last injection

These observations find their explanation in the fact that dye, absorbed from the intestine into the lymph with the fat and into the portal blood with the bile, again enters into the intestine with the bile Thus a closed circulation of the dye is established and it is possible that the blood of a once Sudan-stained animal may become quite free from the stained fat only after long periods under normal conditions of feeding Animals examined months after the Sudan-stained feeding had ceased contained deeply stained fatty tissue

The time required for the elimination of circulating fat-soluble dyes

An attempt was made to ascertain (in cats and dogs) the length of time necessary for the separation of the dye from the circulating fat and its elimination through the bile Emulsions of stained fat, in amounts varying from 1 to 10 cc, were injected directly into the circulation, cannulae were placed in the common bile ducts and samples of blood and bile were taken every two or three hours In order to facilitate the flow of bile, solutions of desiccated ox bile were injected into the upper intestine The bile, blood, and liver tissue after it had been washed free from blood, so far as possible, were examined for the stain

Nine and one-half hours was the longest period during which observations were made in any experiment, and although in that instance only 1 cc of the stained emulsion was injected, both blood and bile, collected at the end of this time, showed that a considerable quantity of stained fat was still in circulation In those cases in which the experiments continued over a comparatively long time, or when a small amount of the stained fat had been introduced, the liver was free from the stain The liver evidently does not store up stained fat, the dye becomes separated from the fat as the stained fat comes in contact with the bile in the liver cells

Discussion Fat-soluble dyes introduced into the body in solution in fat are secreted in the bile These dyes may enter the

body from the alimentary tract in two ways: (1) in the lymph, in solution in fat, (2) through the portal circulation, dissolved in reabsorbed bile. When the dyes are absorbed dissolved in bile, they apparently do not pass beyond the liver, but are speedily reexcreted into the gut, and do not enter the general circulation unless fat is present in the intestine. The blood of Sudan-stained animals, under normal conditions of feeding, is never free from the fat stain. The dye put out in the biliary secretion is reabsorbed in the digesting fat, and a continuous circulation from gut to blood and return is established. The elimination of the stain from the circulation, when all possibility of reabsorption is removed, takes place slowly. The stained fat was found in the blood of a cat nine and one-half hours after it had been injected into the femoral vein.

FAT TRANSPORT IN STARVATION AND PATHOLOGICAL CONDITIONS: PHOSPHORUS AND PHLORHIZIN POISONING

We have attempted to follow the migrations of Sudan-stained fats under conditions in which a transport of fat is well-known to occur, namely, in starvation and after poisoning with phosphorus or phlorhizin. The experimental animals were fed in advance for a period of three to five weeks on Sudan-stained food. Phosphorus was administered subcutaneously, dissolved in oil, phlorhizin similarly in solution in sodium carbonate. Other details of selected protocols are summarized in tabular form.

Sudan III in pathological fat transport and starvation

	ANIMAL	DURATION OF EXPERIMENT	FAT CONTENT OF LIVER	STAIN IN	
				Bile	Blood
		days	*per cent*		
Starvation	Cat I, 13	5	56.0	Present	Present
	Cat I, 18	5	33.5	Present	Present
	Guinea pig	1	7.9		Present
Phosphorus poisoning	Cat XI, 23	9	64.3	Present	Present
	Hen X, 20	4	59.0	Present	
	Hen XI, 29	19	40.5	Present	
Phlorhizin poisoning	Cat XII, 6	12	15.8	Present	Present
	Cat XI, 19	7	11.1	Present	Present

Neither in the foregoing nor in numerous other comparable experiments in which a transport of fat (fatty infiltration) was induced, was any evidence obtainable of dye in the extracts of the liver tissue or in frozen sections thereof The constant finding of the Sudan III in both the blood and bile makes it evident that the dye migrates from the stained adipose tissue and is brought to the liver where it is eliminated in the bile The observations give an additional indication that the fatty livers in these pathological conditions are produced by infiltration of fat, for it is difficult to believe that, if the high content of liver fat had been obtained by a degeneration process in the hepatic tissue, such an accumulation of dye in the bile would have taken place

FAT TRANSPORT TO THE EMBRYO

The question of the origin of foetal fat has been much debated,[22] It involves the broader problem of the passage of substances through the placental barrier S H and S P Gage ('08) failed to find the adipose tissues of the young stained, when stained fats were fed to pregnant mothers Hofbauer ('05) believed that he found particles of dye in the foetal blood and assumed that they had become separated from fat metabolized by the embryo His method—microscopic examination—is scarcely adapted to determine this point, however

Numerous experiments in which we have fed rats and cats with Sudan-stained food or Biebrich Scarlet throughout the period of gestation have uniformly shown *an absence of the dye in the foetus or the newly-born young* Two illustrative protocols, selected from many similar ones, will suffice to show our method of investigation

Rat C Sudan-feeding was begun sixteen days before the young were born The alimentary tract was removed from one of them soon after birth Its contents were distinctly pink (from mothers milk) The ether extract of the entire residual body was uncolored Subsequent examination of the mother showed deeply stained adipose tissue

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[22] Cf Ahlfeld *Centralbl f Gynackol*, i, p 265, 1877, Thiemich *Centralbl f Physiol*, xii, p 850, 1898, *Jahrb f Kinderheilk*, lxi, p 174, 1905, Hofbauer, J *Arch f Gynaekol*, lxxvii, p 139, 1906, Oshima *Zentralbl f Physiol*, xxi, p 297, 1907, Bondi *Arch f Gynaekol*, xciii, p 189, 1911

Cat B Was fed 80 mgms of Sudan III every second day for eighteen days prior to birth of kittens Aside from the stomach contents there was no pink in the ether extract of tissues of the young examined soon after birth The adipose tissue of the mother was deeply stained

Although it is unlikely from such findings that stained fat can pass through the placenta, this is not necessarily conclusive evidence that the foetal fat has its origin in substances other than fat The findings in the case of the alimentary epithelial tissues and glandular structures however add little likelihood to the transport or deposition of the fat in a non-stainable combination

FAT TRANSPORT INTO MILK

The precise relation of milk fat to food fat and the extent to which the latter can pass directly into the mammary secretion without first becoming a part of the body stores is not easily determined S H and S P Gage ('09) found Sudan III in the milk of rats after prolonged feeding with the dye, this, however, is no proof of the immediate origin of the milk fat from the food, since the fat depots of the rats were also stained In explanation of the observations that foreign food fats have more frequently been found secreted in the milk of smaller animals (goats, sheep, dogs, rats) than of cows, it has been suggested that the milk secretion is more directly dependent upon the food supply in the smaller species[23] However, the marked differences in the time required to stain the adipose tissue of guinea pigs and rabbits with Sudan III in comparison with cats and rats, suggests that the discrepancies noted above may bear some relation to the readiness with which the different animals absorb and store fat

EXPERIMENTAL We have investigated the appearance of Sudan III in the milk after feeding the dye both before and during the period of lactation When animals, notably cats, have refused to eat stained fat, the dye has been administered in capsules either directly before or after a meal rich in fat This fact is important for successful results In the case of cats and rats the character of the milk was determined by examining the stomach contents of suckling young Needless to say great care must be taken to

[23] Cf Lusk *Science of Nutrition*, 1909, p 237

have the cages scrupulously free from stained food which might lead to erroneous conclusions

It is scarcely necessary to repeat here the details of the many trials, since the methods are fairly obvious Both Sudan III and Biebrich Scarlet were found to be secreted into the milk by rats, Sudan excretion was likewise observed in cats, guinea pigs and a goat In the case of the goat, one gram of Sudan III dissolved in oil was added to the feed twice daily during six successive days [24] The milk drawn nine hours after the first dose showed the presence of the dye, the tint increasing with the subsequent milkings The guinea pigs received 2 cc of stained olive oil every other day

An important fact in this connection is the observation that the color disappears from the milk when the Sudan-feeding is discontinued, despite the persistence of the stain in the adipose tissues of the secreting animals This was likewise true in experiments with Biebrich Scarlet

The following protocol illustrates the transport of storage stained fat during starvation and the passage of the dye into the milk

Rat 11 Was fed Sudan-stained food during the period of gestation Soon after the birth of the young, April 30, the cage was cleaned and unstained food thenceforth employed On May 14 the ether extract of milk found in the stomach of a suckling rat was uncolored The mother was now starved two days At the end of this time the milk in the stomach of another one of the young gave a faintly pink ether extract The adipose tissue of the adult was found to be stained still This experiment was duplicated with another mother

Like S H and S P Gage, we have failed to induce the secretion of Sudan III in the milk of cows A Holstein cow was given 7 5 grams, twice daily, dissolved in olive oil and added to the mash feed on three successive days, without positive results In considering this we recall that the milk of the goat and guinea pig—animals in whose diet fat likewise plays a comparatively small rôle—was decidedly faint in color in comparison with the milk of cats and rats Bearing in mind the necessity of fat for the transport of the dye an explanation at once suggests itself for the inequalities here observed

[24] This experiment was conducted at the New York Agricultural Experiment Station in Geneva, through the kindness of Director W H Jordan

SUMMARY

Some of the fat-soluble dyes, introduced into the organism by various paths, are deposited in the adipose tissues and bone marrow The renal and nervous tissues are free from the stain, even when the fatty tissues are deeply colored Muscle probably does not take up the dye It is seldom found in the liver, because the fat-soluble dyes, which are insoluble in water, dissolve readily in the bile and are excreted thereby into the intestine from which they can be reabsorbed

The fat-soluble dyes may enter the organism from the alimentary tract through the lymphatics, in solution in fat, or by the portal circulation, dissolved in reabsorbed bile They do not pass beyond the liver unless fat is present to transport them Then they may be found in the blood, which is rarely free from the dye in a normally fed animal that has once been stained A cycle between intestine, bile and blood becomes established No elimination of the dyes occurs through the kidneys, except when an alimentary lipuria arises (in rabbits and rats)

Contrary to the assertion of others, the stained fat is no less available to the organism than the unstained

In cases conducive to fat transport—in starvation, phosphorus and phlorhizin-poisoning—stained fat migrates from the stained depots to the blood and the liver cells Here the dye is separated and secreted into the bile, so that the liver, though having a high content of fat, may be free from the dye

Stained fat does not traverse the placenta The blood of the foetus and the fat of young born of Sudan-stained mothers is free from dye

The excretion of Sudan III and Biebrich Scarlet in milk, when they are given with food fat, suggests that the latter may pass directly into the mammary secretion With cats and rats the results are striking, but the dye excretion in milk ceases when the stained food is no longer fed In guinea pigs and goats the secretion of dye in the milk is positive, in the cow it has not yet been demonstrated The variation in the outcome in the different species may be due to variations in the relative abundance in the dietaries of fat necessary for the absorption and transport of the dye This explanation is emphasized by the observation that those

animals (cats, rats, hens, pigeons) for which fat enters more largely into the diet, become stained more easily or speedily than animals which are accustomed to ingest relatively smaller amounts of fat

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SOME NEW COMPOUNDS OF THE CHOLINE TYPE. II.

CERTAIN ACYL DERIVATIVES OF α-METHYLCHOLINE, "β-HOMOCHOLINE" (β-METHYLCHOLINE) AND γ-HOMOCHOLINE[1]

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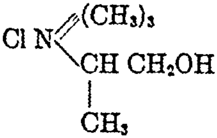
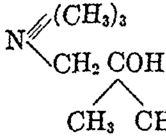
(Received for publication, August 31, 1912.)

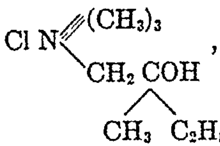
In a preliminary paper[2] under the same general title, describing the chlorides of α-methylcholine,



, β-dimethylcholine,



and β, β-methylethylcholine,



, the writer announced that certain acyl derivatives of α-methylcholine had also been prepared. The description of these compounds, however, was deferred at that time, pending the completion of certain minor details of investigation essential thereto. These experimental details have long since been completed but the published description of the compounds has been still further delayed by the demands of other work. Such description is here submitted together with that of several other acyl derivatives and certain of their salts.

A number of acyl derivatives of α-methylcholine chloride have been prepared. In all cases, except that of the acetyl derivative,

[1] Submitted, in abstract, to the International Congress of Applied Chemistry, New York, September, 1912.

[2] This *Journal*, x, pp. 399–406.

alcohol but soluble in water and in mixtures of alcohol and water It melts[3] with decomposition at about 222°–223° (corrected) Analysis for platinum by ignition resulted as follows

0 1535 gram salt gave 0 0411 gram Pt

	Calculated for $C_{16}H_{36}O_4N_2Cl_6Pt$	Found
Pt	26 70	26 77

The gold salt, $\left(N \begin{cases} \equiv (CH_3)_3 \\ -CH\,CH_2OCOCH_3 \\ \quad | \\ \quad CH_3 \end{cases}\right) AuCl_4$, was prepared in a manner similar to the platinum salt just described and also by adding a concentrated aqueous solution of chlorauric acid to a concentrated aqueous solution of the acetyl derivative In each case the salt separated as a pale yellow, crystalline solid Under the conditions outlined in a previous footnote it melts at 124°–125 5° (corrected) Analysis for gold by ignition in the two cases resulted as follows

I 0 1459 gram salt gave 0 0574 gram Au
II 0 1373 gram salt gave 0 0540 gram Au

	Calculated for $C_8H_{18}O_2NCl_4Au$	Found I	Found II
Au	39 48	39 34	39 33

Benzoyl derivative of α-methylcholine chloride (benzoyl-β-methylethoxytrimethylammonium chloride), $Cl\,N \begin{cases} \equiv (CH_3)_3 \\ -CH\,CH_2OCOC_6H_5 \\ \quad | \\ \quad CH_3 \end{cases}$

This compound was prepared by heating the α-methylcholine chloride with a large excess of benzoyl chloride, in a flask with reflux condenser, to 100° (water bath) for about two hours The reaction product poured into dry ether yielded an oily precipitate

[3] All "melting points" here reported were determined by the usual capillary-tube method in a bath of sulphuric acid, using standardized Anschutz thermometers for which a correction for emergent stem was not considered necessary The rate of heating in all cases was regulated so as to closely approximate 5° a minute within 20° to 30° of the melting point As pointed out in Hygienic Laboratory Bulletin 70, and as corroborated in this and other work, the *decomposition* point may vary quite widely with comparatively slight variation in manipulation

which was worked up in the same manner as the acetyl derivative except that the last traces of benzoyl chloride were removed by dissolving the oily precipitate in water and extracting several times with ether The aqueous layer was then evaporated to dryness in a vacuum desiccator, containing concentrated sulphuric acid, yielding a white, crystalline, hygroscopic solid The reaction was apparently neither so smooth nor so nearly quantitative as for the acetyl derivative

The platinum salt of benzoyl-α-methylcholine,

$$\left(\begin{array}{l} N \equiv (CH_3)_3 \\ \;\diagdown CH\ CH_2OCOC_6H_5 \\ \qquad | \\ \quad\ CH_3 \end{array}\right)_2 PtCl_6,$$

was obtained by dissolving a portion of the above described solid in a little cold water and precipitating with a cold aqueous solution of chlorplatinic acid It appeared to be quite insoluble in cold water since careful concentration of the mother liquor caused no further separation Heated in a capillary tube the salt begins to sinter definitely and to darken above 233° and decomposes with effervescence at 236 5° to 237 5° (corrected) Analysis for platinum by ignition resulted as follows

0 1178 gram salt gave 0 0270 gram Pt

	Calculated for $C_{26}H_{40}O_4N_2Cl_6Pt$	Found
Pt	22 87	22 92

The gold salt,

$$\left(\begin{array}{l} N \equiv (CH_3)_3 \\ \;\diagdown CH\ CH_2OCOC_6H_5 \\ \qquad | \\ \quad\ CH_3 \end{array}\right) AuCl_4,$$

was not easily obtained, from the quantity available, in condition favorable for accurate analysis It was precipitated from an aqueous solution of the benzoyl-choline derivative with concentrated aqueous chlorauric acid and separated as a pale yellow very viscous oil It is quite soluble in alcohol and slightly soluble in hot water

The phenylacetyl derivative of α-methylcholine chloride (phenylacetyl-β-methylethoxytrimethylammonium chloride),

$$\begin{array}{l} Cl\ N \equiv (CH_3)_3 \\ \quad\;\diagdown CH\ CH_2OCOCH_2C_6H_5 \\ \qquad\quad | \\ \qquad\ CH_3 \end{array},$$

was prepared in the following man-

ner About 1 gram of α-methylcholine chloride was mixed in a Carius tube with a moderate excess of phenylacetylchloride, the tube was sealed, heated in boiling water for about five hours, allowed to stand in the cooling bath over night, and the product poured into dry ether As in previous cases the acyl derivative separated in an oily condition

In order to test the stability of the phenylacetyl group in the molecule a small portion of the oily product was taken up in cold water, extracted with ether, and the aqueous solution evaporated to dryness on a water bath The residue obtained was dissolved in alcohol and precipitated with alcoholic chlorplatinic acid A platinum analysis of the resulting salt indicated, with practically theoretical accuracy, the platinum salt of α-methylcholine, suggesting that either the acylation had not proceeded at all or complete saponification of the ester had resulted from the treatment with water

The remainder of the original oily residue was taken up in cold water, extracted with ether, and a portion of the aqueous solution treated at once with aqueous chlorplatinic acid A yellow, more or less waxy, precipitate separated which proved to be the platinum salt of the phenylacetyl derivative,

$$\left(N \begin{cases} \equiv (CH_3)_3 \\ -\underset{\displaystyle CH_3}{\underset{|}{CH}}\, CH_2OCOCH_2C_6H_5 \end{cases}\right)_2 PtCl_4$$

It is insoluble in absolute alcohol but soluble even in cold water The attempt to concentrate the mother liquor on a water bath, however, resulted in decomposition On heating the salt in a capillary tube it shows the effect, slightly, as low as 219°, turns dark gradually above 229° and decomposes with effervescence at about 245 7°–246 7° (corrected)

Analysis for platinum by ignition resulted as follows

0 1021 gram salt gave 0 0225 gram Pt

	Calculated for $C_{18}H_{44}O_4N_2Cl_6Pt$	Found
Pt	22 14	22 04

As in the case of the benzoyl derivative the gold salt of the phenyl-

acetyl derivative, $\left(N\begin{array}{l}\diagup\!\!\!\diagup (CH_3)_3 \\ \diagdown CH\,CH_2OCOCH_2C_6H_5 \\ \quad\;|\\ \quad\;CH_3\end{array}\right)AuCl_4$, separates as an oil and could not readily be obtained in a condition favorable for melting point determination and analysis It is readily soluble in hot water and slightly soluble in 95 per cent alcohol but appears to undergo decomposition in both cases as shown not only by analysis but also by marked change in melting point One sample of the salt, undoubtedly impure, melted slowly over a wide range from about 140°–160°, but on recrystallizing from hot water (yielding well-formed crystals) it melted at about 192°–194°, which approximates the melting point of the gold salt of α-methylcholine itself, 197°–198 5° (corrected) Similar observations were made on the behavior of the platinum salt It appears therefore that the phenylacetyl derivative of α-methylcholine is not a very stable compound either in the form of the chloride, the platinum salt, or the gold salt

For the purpose of comparison, both physiologically and chemically, with the choline derivative just described the corresponding derivative of "β-homocholine"[4] (β-methylcholine) chloride was prepared

Phenylacetyl-β-methylcholine chloride (phenylacetyl-β-oxypropyltrimethylammonium chloride), $Cl\,N\begin{array}{l}\diagup\!\!\!\diagup (CH_3)_3 \\ \diagdown CH_2\,CHOCOCH_2C_6H_5 \\ \qquad\quad\;|\\ \qquad\quad\;CH_3\end{array}$

This compound was prepared by heating a mixture of the corresponding choline chloride and phenylacetyl chloride (in excess) in a sealed tube to 100° (water bath) for about three hours As with the other preparations described, treatment with dry ether precipitated an oily semi-solid product A portion of this was converted into the platinum salt, analysis of which indicated a mixture of the unaltered β-methylcholine and the desired derivative

An attempt to separate this mixture into its component salts by

[4] Other acyl derivatives of "β-homocholine" have been prepared in this Laboratory and are briefly described by Hunt and Taveau in Hygienic Laboratory Bull No 73, U S Public Health Service

fractional crystallization from hot water was successful Two distinct fractions were obtained The less soluble fraction showed a melting point of about 250°, corresponding to that reported by Hunt and Taveau[6] for "β-homocholine" platinum salt The more soluble (smaller) fraction melted at 216°–217° (corrected), and analysis for platinum indicated it to be the platinum salt of phenylacetyl-β-methylcholine,

$$\left(N \begin{cases} (CH_3)_3 \\ CH_2\,\underset{\displaystyle CH_3}{\underset{|}{C}}HOCOCH_2C_6H_5 \end{cases} \right)_2 PtCl_6,$$

as shown in the following data

0 1052 gram salt gave 0 0233 gram Pt

	Calculated for $C_{32}H_{44}O_4N_2Cl_6Pt$	Found
Pt	22 14	22 15

This experiment demonstrated in a striking way the greater stability of this particular derivative of β-methylcholine as compared with the same derivative of α-methylcholine (described above) under similar conditions, and suggests the possibility of a general rule to the effect that esters of secondary alcohols are less easily saponified than the same ester of isomeric primary alcohols

A second preparation of phenylacetyl-β-methylcholine, involving the same quantities of reagents as in the first, was made in a successful attempt to obtain a more quantitative reaction The mixture in a sealed tube was heated in a boiling water bath for about seven hours It was then transferred to a tube-furnace and heated to 150° for about one hour The reaction product, treated as in previous preparations, was converted into the platinum salt, which without recrystallization analyzed as follows

0 1076 gram salt gave 0 0238 gram Pt

	Calculated for $C_{32}H_{44}O_4N_2Cl_6Pt$	Found
Pt	22 14	22 03

The gold salt of this derivative,

[6] Hygienic Laboratory Bull No 73, U S Public Health Service, footnote, p 33

$\left(N\begin{matrix}(CH_3)_3 \\ CH_2\,\underset{\displaystyle CH_3}{\underset{|}{C}}HOCOCH_2C_6H_5\end{matrix}\right)AuCl_4$, was also prepared, using chloride of the base that had been standing in a vacuum desiccator for about four weeks On adding chlorauric acid to an aqueous solution of the choline chloride the gold salt separated as a pale yellow oil which quickly became crystalline on rubbing On heating in a capillary tube it sintered slowly above 60°, fused without flowing at 74 5°–76°, became limpid and fairly clear at about 85° (not corrected) Analysis for gold by ignition resulted as follows

0 1080 gram salt gave 0 0374 gram Au

	Calculated for $C_{14}H_{22}O_2NCl_4Au$	Found
Au	34 28	34 63

From the aqueous mother liquor on standing a considerable further separation of flaky crystals resulted In a melting point determination this fraction showed the same general behavior as the crystallized oil, but at a higher temperature, fusion to a mushy mass occurring at 86 5°–88 5° instead of at 74 5°–76° Analysis disclosed the considerably higher gold content of 36 79 per cent The crystallized oil fraction, which was slightly soluble in cold water and in cold absolute alcohol but readily soluble in these solvents hot, was dissolved in a little hot water from which on cooling it separated in flaky crystals This fraction showed a further difference in melting point, fusing at 114 5°–115 5° and becoming a clear limpid liquid at 118° (uncorrected) The remaining material was insufficient for analysis

In view of the data derived from the platinum salt of this derivative the above described behavior of the gold salt would suggest decomposition of the gold salt rather than serious impurity in the original product, though there is the alternative possibility (which was not verified) of partial decomposition of the chloride of the base on long standing in a vacuum desiccator

For comparison with the phenylacetyl derivatives already described the corresponding derivative of "γ-homocholine"[6]

[6] Other acyl derivatives of "γ-homocholine" have been prepared in this laboratory and are briefly described by Hunt and Taveau in Hygienic Laboratory Bull No 73, U S Public Health Service

$Cl\,N\begin{array}{l}\diagup(CH_3)_3\\ \diagdown CH_2\,CH_2\,CH_2OCOCH_2C_6H_5\end{array}$, was also prepared This compound was prepared by the same method applied to the β-compound Only two hours' heating in the water bath, however, appeared sufficient to induce complete reaction As in other cases the crude reaction product was oily but on concentrating, in a vacuum desiccator, a portion of the aqueous solution, after extracting with ether, it gradually separated as a white flaky solid Another portion of the aqueous solution was precipitated with chlorplatinic acid forming the platinum salt,

$\left(N\begin{array}{l}\equiv(CH_3)_3\\ \diagdown CH_2\,CH_2\,CH_2OCOCH_2C_6H_5\end{array}\right)_2PtCl_6$ This salt is practically insoluble in alcohol and difficultly soluble in hot water from which it crystallizes in individual, and clusters of, stocky prisms It melts sharply with decomposition at about 193° to 194° (corrected) On ignition

0 1031 gram salt gave 0 0230 gram Pt

	Calculated for $C_{28}H_{44}O_4N_2Cl_6Pt$	Found
Pt	22 14	22 31

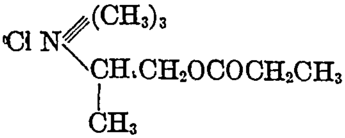
The gold salt of this derivative,

$\left(N\begin{array}{l}\equiv(CH_3)_3\\ \diagdown CH_2CH_2CH_2OCOCH_2C_6H_5\end{array}\right)AuCl_4$, was precipitated from aqueous solution with aqueous chlorauric acid and separated as a pale yellow, crystalline solid It is nearly insoluble in cold water and rather difficultly soluble in warm water from which on cooling it separates in flaky crystals The solubility in absolute alcohol is considerably greater than in water It melts slowly with slight effervescence at 129°–131° (corrected) On ignition

0 1073 gram salt gave 0 0370 gram Au

	Calculated for $C_{14}H_{22}O_2NCl_4Au$	Found
Au	34 28	34 48

The propionyl derivative of α-methylcholine chloride (propionyl-β-methylethoxytrimethylammonium chloride),

, was prepared by the same method and procedure already fully described Analysis of the crude gold and platinum salts indicated that the reaction was still not quite complete after heating the mixture of α-methylcholine and propionyl chloride (in excess) in a sealed tube for between three and four hours in a boiling water bath No attempt was made to purify the gold salt, analysis of which, however, indicated a sufficient degree of purity for preliminary pharmacological purposes as shown in the following data

0 2 gram salt gave 0 0776 gram Au

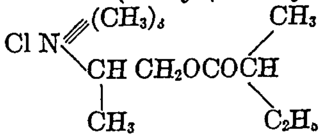
	Calculated for $C_9H_{20}O_2NCl_4Au$	Found
Au	38 43	38 8

The platinum salt was purified by recrystallizing from hot water The pure salt decomposes, under the conditions previously outlined (footnote 3), at about 231°–232° (corrected) On ignition for platinum analysis

0 1101 gram salt gave 0 0285 gram Pt

	Calculated for $C_{18}H_{40}O_4N_2Cl_6Pt$	Found
Pt	25 77	25 88

Valeryl, monobromisocapronyl, and palmityl derivatives of α-methylcholine were also prepared by the method described

The valeryl derivative (valeryl-β-methylethoxytrimethylammonium chloride,  was obtained in pure form without difficulty The platinum salt, precipitated from aqueous solution, on heating in a capillary tube gradually sinters above 219° and decomposes with effervescence at about 228°–229° (corrected) Ignited for platinum

0 1526 gram salt gave 0 0367 gram Pt

	Calculated for $C_{22}H_{48}O_4N_2Cl_6Pt$	Found
Pt	23 99	24 05

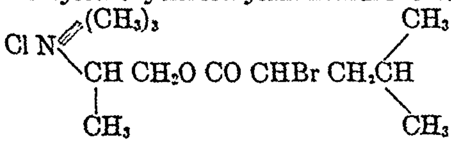
The gold salt separated as an oil from both aqueous and alcoholic solution On standing over night at room temperature it crystallized (prisms) Heated in a capillary tube it sinters sharply at 72° and is completely melted on slowly heating to 75° It is practically insoluble in cold water, slightly soluble in cold absolute alcohol and in hot water, and readily soluble in hot alcohol Analysis for gold resulted as follows

0 1224 gram salt gave 0 0449 gram Au

	Calculated for $C_{11}H_{24}O_2NCl_4Au$	Found
Au	36 44	36 68

Subsequent investigation of the valeryl derivative, by means of platinum and gold preparations, indicated that it had deteriorated on long standing in a vacuum desiccator

The monobromisocapronyl derivative (α-bromisocapronyl-β-methylethoxytrimethylammonium chloride),



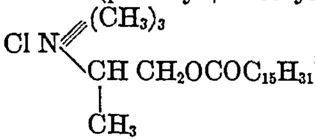
was obtained without serious difficulty, though perhaps not perfectly pure, after about five hours' heating of the reacting mixture in the water bath The platinum salt, freshly precipitated from aqueous solution, appeared of waxy consistency but became more granular on rubbing with a glass rod Precipitated from alcoholic solution it was still more waxy in character It is practically insoluble in absolute alcohol, cold or hot, and in cold water, but dissolves slowly in hot water from which on cooling it separates in a dense flocculent mass, isolated patches of which appeared, under the microscope, to be clusters of very fine needles The clean dry salt, heated in a capillary tube, decomposes at about 226°–227° (corrected) On ignition for platinum

0 1507 gram salt gave 0 0291 gram Pt

	Calculated for $C_{24}H_{50}O_4N_2Cl_6Br_2Pt$	Found
Pt	19 52	19 31

The gold salt of this derivative, which separates from aqueous solution (and persists) as a reddish oil, could not be obtained in sufficient purity to give a satisfactory analysis

A first attempt to prepare the palmityl derivative of α-methylcholine (palmityl-β-methylethoxytrimethylammonium chloride),

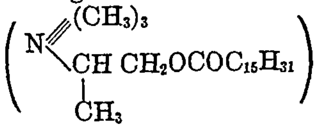


,resulted, as determined by platinum salt, in absolute failure The experiment was repeated taking special precautions to use dry reagents The mixture, including a larger excess of palmityl chloride than in the first attempt, was heated in a water bath for about six hours and allowed to remain sealed and standing in the cooling bath over night On pouring the reaction product into dry ether a finely divided white waxy solid, more or less oily, separated This proved to be so much less soluble in water than the other acyl derivatives that preparation of the platinum salt from aqueous solution was impractical Analysis of the platinum salt, precipitated from alcoholic solution, indicated the reaction product to be a mixture of unaltered α-methylcholine chloride and the palmityl derivative The salt was treated with hot water and heated with stirring on a water bath A comparatively small part of it dissolved readily, the rest remaining apparently unaffected even on the addition of more water The dissolved portion, largely recovered by concentration of the mother liquor, gave analytical results for platinum which were practically theoretical for the platinum salt of the unaltered base The undissolved portion proved to be, as indicated by analyses for platinum, the platinum salt of the palmityl derivative It is practically insoluble in alcohol and in water, cold or hot Heated in a capillary tube it decomposes with effervescence at about 240°–241° Duplicate analyses for platinum by ignition resulted as follows

I 0 1005 gram salt gave 0 0172 gram Pt
II 0 1006 gram salt gave 0 0173 gram Pt

	Calculated for $C_{44}H_{92}O_4N_2Cl_6Pt$	Found I	Found II
Pt	17 39	17 12	17 2

The gold salt of the palmityl derivative,



$AuCl_4$, was also prepared but was not

easily obtained pure It is too soluble in absolute alcohol to be prepared in that medium or to be recrystallized from it, but when water is added to the alcoholic solution the salt separates as a pale yellow greasy precipitate, which was made more definitely crystalline by washing with ether It is readily soluble in absolute alcohol, slightly soluble in water and in ether It melts (not sharply) at about the same temperature as the gold salt of the valeryl derivative (72°–75°) A large part of available salt was lost by decomposition in attempting to dry it in an air bath at about 80° The small remainder was more carefully dried, and analyzed for gold by ignition (to constant weight)

0 0980 gram salt gave 0 0275 gram Au

	Calculated for $C_{22}H_{44}O_2NCl_4Au$	Found
Au	28 37	28 06

Since the publication of the preliminary paper cited above the development of new compounds of the choline type has been continued in this laboratory, as opportunity permitted, with more or less success Some of the results obtained in this later work will be submitted for publication at an early date

A STUDY OF THE TRYPTIC PROTEOLYSIS OF CYNOSCION REGALIS

By GEORGE F WHITE AND ADRIAN THOMAS

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(Received for publication, September 3, 1912)

It is frequently desired to compare the rate and course of digestion of various proteins by enzymes, according to methods which while not complicated will give distinctive and reliable results Van Slyke's[1] method for the determination of amino-acids has been successfully applied by White and Crozier[2] to a comparison of the tryptic proteolysis of beef and several fish meats The ease of manipulation of the apparatus, the brief time required for a determination and the regularity of the experimental data, makes the process generally useful Also the conclusions drawn conform with those from metabolism experiments, making the results of still greater value

The method which Sorensen[3] has proposed for the estimation of amino-acids and polypeptides, by titration with caustic soda after the addition of formaldehyde, by its simplicity suggests itself for artificial digestion processes It is the object of this article to show its application to the hydrolysis of *Cynoscion regalis* (squeteague, weakfish) by trypsin, and to compare the results with those obtained by Van Slyke's method

The squeteague was boiled in water for one-quarter of an hour, allowed to drain from excess of liquid and preserved ice-cold An analysis of two samples gave an average of 4 52 per cent N

The digestion was carried on in 250 cc volumetric flasks placed in a thermostat kept at a temperature of 37 5°C Enough meat to furnish 1 5 grams of nitrogen was weighed out, ground up with water together with

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[1] This *Journal*, ix, p 185, 1911

[2] *Journ Amer Chem Soc* , xxxiii, p 2042, 1911

[3] *Biochem Zeitschr* , vii, p 45, 1907

1 gram of trypsin, and this mixture poured into the flask. Twenty-five cubic centimeters of $\frac{N}{10}$ sodium hydroxide solution were added and the whole made up to 250 cc. with water. A little chloroform was then added. Trypsin is presumably most active in a medium which is alkaline with sodium carbonate, but the presence of this salt would interfere with the titration for the amino-acids where phenolphthalein must be used as an indicator, so that the alkalinity was ensured by the addition of the hydrate. Separate mixtures were made and analyses run in duplicate for the time periods of one, one-half, two, five and eight hours respectively. A sample, similar to the above but containing no trypsin, was also prepared and analyzed with the others according to the following method.

At the end of the desired time of digestion, the mixtures were filtered and aliquot portions of the filtrate taken for the various tests. Ten cubic centimeters were used for the determination of total soluble nitrogen by the Kjeldahl method, 10 cc. for the amount of amino nitrogen by Van Slyke's method and 20 cc. were treated with 10 cc. of 40 per cent formaldehyde solution and titrated to a distinct pink color with $\frac{N}{10}$ sodium hydroxide solution. We found that in every case an excellent end point was obtained, duplicate analyses agreeing within 0.2 per cent. Finally, 10 cc. of the filtrate from the digested fish were completely hydrolyzed by prolonged digestion on the water bath with 40 cc. of concentrated hydrochloric acid. This solution was evaporated to dryness, made up to 50 cc. with water, 10 cc. tested for amino nitrogen by Van Slyke's method and 20 cc. analyzed for amino-acids by Sörensen's method.

All the results obtained as above were corrected for amino nitrogen before and after complete hydrolysis, for total soluble nitrogen and for amino-acids as determined by titration with caustic soda, by carrying through the same experiments with the trypsin and alkali but with no protein. Correction was also made for the alkali required to neutralize the formaldehyde solution.

TABLE I

Total and amino nitrogen in solutions of Cynoscion regalis Hydrolyzed by trypsin

TIME IN HOURS	SOLUBLE N	INSOLUBLE N	AMINO N	AMINO N AFTER HYDROLYSIS BY HCl	AVERAGE SIZE OF PEPTIDES	$100 \times \frac{\text{SOLUBLE N}}{\text{TOTAL N}}$	$100 \times \frac{\text{AMINO N}}{\text{SOLUBLE N}}$
0	0.170	1.330	0.017	0.056	3.29	11.32	10.00
½	1.115	0.385	0.230	0.464	2.02	74.32	20.63
1	1.175	0.340	0.250	0.464	1.86	78.34	21.27
2	1.173	0.327	0.289	0.464	1.61	78.20	24.63
5	1.361	0.139	0.357			90.72	26.23
8	1.432	0.068	0.406	0.646	1.59	95.45	28.35

In table I are presented the results obtained by applying Van Slyke's method to the tryptic proteolysis of squeteague The average size of the peptides was calculated by dividing the amount of amino nitrogen present after complete hydrolysis with hydrochloric acid by that in the solution before such hydrolysis The last two columns of data show the increase with time of the proportion of soluble to insoluble nitrogen and of amino to total soluble nitrogen respectively

The average results of duplicate analyses are given

TABLE II

Analysis by Sörensen's method of solutions of Cynoscion regalis Hydrolyzed by trypsin

TIME IN HOURS	CC $\frac{N}{10}$ NaOH REQUIRED	CC $\frac{N}{10}$ NaOH REQUIRED AFTER HYDROLYSIS WITH HCl	AVERAGE SIZE OF PEPTIDES	AMINO N CALCULATED	$100 \times \frac{\text{AMINO N CALCULATED}}{\text{SOLUBLE N}}$
0	24 85	125 0	5 02	0 035	20 52
½	182 8	536 8	2 94	0 257	23 02
1	213 8	598 0	2 80	0 300	25 87
2	232 9			0 327	27 87
5	293 5	776 0	2 64	0 412	30 28
8	314 8	787 1	2 50	0 442	30 87

In table II are given the average results of the analysis of the proteolyzed solutions according to Sorensen's method In column 2 the figures represent cubic centimeters of $\frac{N}{10}$ sodium hydroxide solution required for neutralization after addition of formaldehyde solution Column 3 is the same for the solutions after complete hydrolysis with hydrochloric acid The next column gives the ratio of the latter to the former In column 5 are given figures for amino nitrogen calculated from the data in column 2, while in the last column the values of the calculated amino nitrogen are expressed as percentages of the total soluble nitrogen

From table I it is seen from the ratio of the soluble to total nitrogen that the fish meat goes very rapidly into solution, 74 32 per cent of the nitrogen being in solution at the end of one-half hour's digestion Solution, however, is not complete in eight hours'

time, a fact which is *apparently* not in harmony with the results of White and Crozier,[4] who found with the proteins they studied that all the nitrogen was in the soluble form in from four to eight hours The trypsin used, a commercial sample, was of the same activity in both series of experiments These latter experiments were carried on in a medium made alkaline with sodium carbonate, while the experiments described in this article required sodium hydroxide for reasons already stated Schierbeck[5] has proved that the action of trypsin in digesting proteins is accelerated by the presence of carbon dioxide in solutions which are slightly alkaline,

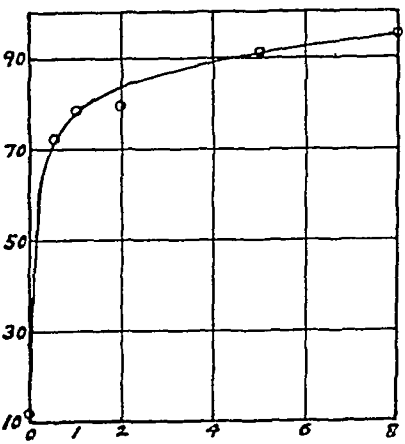

FIG 1 Abscissae—Time in hours
Ordinates—$100 \times \frac{\text{Soluble Nitrogen}}{\text{Total Nitrogen}}$

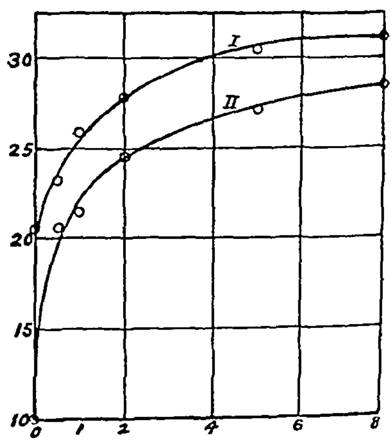

FIG 2 Abscissae—Time in hours
Ordinates—$100 \times \frac{\text{Amino Nitrogen}}{\text{Soluble Nitrogen}}$
I By Sorensen's method
II By Van Slyke's method

and it is very probably at least due partly to this fact that the above differences are found The variation of the proportion of soluble to total nitrogen with increase of time is shown graphically in figure 1 Extrapolation of the curve would indicate that the squeteague would be completely dissolved in about fourteen hours

White and Crozier have shown that their artificial digestion experiments gave results agreeing closely with those obtained from

[4] *Loc cit*
[5] *Sl and Arch f Physiol*, III p 344, 1892

metabolism work with dogs, rates of digestion of different proteins being in the same relation to each other Van Slyke and White,[6] in a study of the relation between the digestion and retention of ingested proteins, found that squeteague is digested more slowly than either beef or cod From the above facts, it is fair to conclude that the tardy solution of the squeteague by trypsin, shown by our data, is not alone due to the absence of carbon dioxide, but is a consequence of the inherent nature of the protein itself

The amino nitrogen in solution increases of course with length of time The average size of the peptides split off from the protein should be especially noted From these data and the experiments of White and Crozier it is evident that the proteins studied break down into simple cleavage products practically *as soon as they go into solution* At the end of one-half hour's digestion the average size of the peptides is only 2 02—the cleavage products on the whole are indicated to be amino-acids The cleavage of certain proteins by trypsin has been intimately studied, and it is known that some amino-acids are readily formed while others are produced very slowly or not at all The above results, however, show that the greater portion of the amino nitrogen in solution exists in bodies of exceedingly simple character The significance of this, physiologically, cannot be pointed out here but will be reserved for future discussion

The relation of the amino to the total soluble nitrogen is shown in figure 2 After eight hours' digestion only 28 35 per cent of the nitrogen is in the amino form This is a confirmation of earlier work which, as just mentioned, has shown that there are certain substances which resist the hydrolytic action of trypsin altogether The increase in the proportion of amino nitrogen during the eight hours' digestion is seen to be very slight, and we must conclude therefore that cleavage of the meat, while yielding amino bodies of a simple nature, leaves the greater part of the soluble nitrogen combined in substances which are extremely stable

The same conclusions may be drawn from the results of our experiments involving Sorensen's method In figure 2 the curves are of the same slope, although of course only approximate figures are expected on assuming the presence of one amino group for

[6] This *Journal*, ix, p 226, 1911

each carboxyl group indicated by the sodium hydroxide required The amino nitrogen thus estimated is regularly greater than that directly determined by the nitrous acid method, and the peptides as computed are about 1 5 times as large It is possible that in such calculations, this effect is produced by the presence of such monamino-dicarboxylic compounds as glutamic acid The discrepancy in the results is not of such a magnitude as to prevent deducing rigid conclusions concerning the rate and general course of digestion of such proteins as the one under investigation

SUMMARY

1 Sorensen's method for the determination of amino-acids was applied to a study of the tryptic digestion of *Cynoscion regalis* The results were found to be regular and in accord with those obtained by the nitrous acid method of analysis for amino nitrogen

2 The relatively low rate at which the protein is made soluble agrees with the results of metabolism experiments

3 Very low cleavage products are formed as soon as the protein goes into solution, the average size of the peptides being 2 02 after one-half hour's digestion

4 There is a very stable nitrogen complex which is not attacked by trypsin

STUDIES ON THE FORMATION OF GLYCOCOLL IN THE BODY II

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(Received for publication, September 6, 1912)

Benzoic acid, circulating in the fluids of the body, combines with glycocoll and removes this intermediary product of protein metabolism from further decomposition The extent to which the elimination of glycocoll can thus occur, depends upon the glycocoll which is available, the amount of benzoic acid present and the rapidity with which the hippuric acid synthesis takes place There is no reason to believe that a reserve of free or preformed glycocoll, sufficient to account for the quantities of hippuric acid eliminated in the urine, exists in the body The apparent independence of the process of glycocoll formation from the general nitrogenous metabolism in the body under certain conditions (such as pure carbohydrate feeding), which we have demonstrated in our previous investigation,[1] points strongly to the conclusion that most of the glycocoll obtained as hippuric acid is the result of a synthetic production of the substance in the body

The attempts made by a number of investigators to cause the synthesis of glycocoll in the body from simpler substances have thus far failed On the other hand, in the absence of such evidence, the facts at our disposal also justify the belief that glycocoll can arise from the higher amino-acids Magnus-Levy[2] regards leucine as being capable of conversion into glycocoll

He concludes by computation on a theoretical basis that most of the glycocoll obtained in benzoic acid experiments can arise

[1] Epstein and Bookman this *Journal*, x, p 353, 1911

[2] Magnus-Levy *Münch med Wochenschr*, lii, p 2168, 1905

from the leucine radical of protein Free leucine however does not yield glycocoll[3] It is only when given as a benzoyl compound that it appears to be entirely converted into glycocoll This deduction is based principally upon two facts, namely, that practically no benzoyl leucine appears, as such, in the urine after administration of large amounts of the substance and that other benzoylized amino-acids do not lead to the production of hippuric acid[4]

If we accept these facts then we must at once conclude that leucine as such does not appear as an intermediary substance in the course of glycocoll production, and that the leucine-bearing portion in protein undergoes a different form of decomposition in the presence of benzoic acid than that which leads to the liberation of leucine itself This gains support from the facts elicited in our previous study that in the course of the production of glycocoll, following the administration of benzoic acid, there does not occur any massive decomposition of protein comparable with that which accompanies phosphorus poisoning and the consequent liberation of leucine and other amino-acids

In brief, if the leucine radical is capable of yielding glycocoll in response to benzoic acid, it must be by some process which does not affect the other protein radicals to any appreciable extent Under the conditions of experimentation recorded by Magnus-Levy, it seems possible that the increased glycocoll output as hippuric acid,[5] following the administration of benzoyl leucine, is not the result of a direct conversion of leucine into glycocoll Benzoic acid does not couple leucine in the body The leucine however is decomposed The effect of a primary benzoylization *in vitro* upon leucine may therefore be such as to render it subject to complete decomposition in the body into substances which are ultimately synthesized into glycocoll The final product (glycocoll) must then be regarded as the result not only of a cleavage process but of a synthesis as well On the other hand, it is also conceivable that the leucine of the benzoyl compound undergoes complete decomposition without taking part in the production

[3] Magnus-Levy *loc cit*, Cohn *Archiv f exp Path u Pharm*, xlviii, p 177, 1902

[4] Magnus-Levy *loc cit*

[5] Magnus-Levy *loc cit*

of glycocoll, and the benzoyl radical which is set free is coupled with glycocoll furnished by the body in much the same manner as benzoic acid

It was with the object of inquiring into this particular question that the present investigation was made To determine this, three sets of experiments were performed In the first of these, various amounts of benzoic acid were administered to rabbits, after the maximum output of hippuric acid was established, leucine was given subcutaneously and the effect upon the production of hippuric acid was studied

Condensed Table 1 Daily average for each period

PERIOD	BENZOIC ACID	LEUCINE N	URINE			
			Total N	Hippuric N	Hippuric N	Extra N
	grams	*gram*	*gram*	*gram*	*per cent*	*gram*
I	0	0	0 428	0 018	4+	
II	2 087	0	0 705	0 135	19	0 142
III	2 087	0 100	0 784	0 118	15	0 238
IV	*0*	*0*	*0 524*	*0 020*	4−	*0 076*

The results obtained in these experiments are in accord with those of Magnus-Levy[6] and of Cohn [7] Leucine evidently does not increase the glycocoll output It appears that the amount of hippuric acid eliminated in the leucine period (III, table 1) is considerably less than that in the control period (II, table 1) The leucine nitrogen appears as extra nitrogen, but not as leucine free leucine could not be detected in the urine

In the second set of experiments, rabbits were given definite amounts of benzoic acid and the maximum hippuric acid output was established, then the dose of benzoic acid was increased and the effect of this increase upon the total hippuric acid output noted Subsequently the animals were given the original quantity of benzoic acid and, in addition, benzoyl-leucine was given in such amounts that the benzoyl radical was the molecular equivalent of the extra dose of benzoic acid

[6] *Loc cit*
[7] *Loc cit*

Condensed Table 2 Daily average for each period

PERIOD	BENZOIC ACID	BENZOYL-LEUCINE		URINE			
				Total N	Hippuric N	Hippuric N	*Extra N*
	grams	*gram*	*gram*	*gram*	*gram*	*per cent*	*gram*
I	0			0 548	0 012	2 5	
II	2 5			0 816	0 108	13	0 160
III	3 125			0 922	0 198	19	0 176
IV	2 5			0 863	0 114	13	0 213
V	2 5	0 519+	0 688	0 868	0 234	27	0 084
VI	2 5			1 442	0 189	13	0 705

On comparing the benzoyl-leucine periods with their corresponding control periods in these experiments we find that a much greater output of hippuric nitrogen occurs in the benzoyl-leucine periods This increment is much greater than the leucine radical of the benzoyl compound could possibly furnish

In the first set (table 2) the hippuric nitrogen of the first benzoic period (II, in which the animal received 2 5 grams of benzoic acid daily) amounts to about 13 per cent of the total nitrogen In the second benzoic period (III, when the dose is increased to 3 125 grams daily) the hippuric nitrogen eliminated comprises 19 per cent of the total nitrogen In other words, the additional 0 625 gram of benzoic acid caused an increase in the hippuric nitrogen output amounting to 6 per cent of the total nitrogen Upon returning to the original dose of benzoic acid (2 5 grams daily) in the third period, the hippuric nitrogen output is again 13 per cent of the total In the following period (V, table 2), when benzoyl-leucine is given in place of the extra 0 625 gram of benzoic acid, the hippuric nitrogen rises to 27 per cent of the total If we allow 19 per cent of this for the benzoic acid equivalent, then there is a balance of 8 per cent nitrogen to the credit of the leucine This is almost the exact equivalent of nitrogen furnished by the leucine (0 094 gram)

Although the results of the second set of benzoyl-leucine experiments differ from the first in a number of details, they point nevertheless to the same conclusion In the first benzoic period (II, table 3) the hippuric nitrogen constitutes 18 per cent of the total eliminated in the urine An additional 0 5 gram of benzoic acid

daily does not seem to cause any greater output of hippuric nitrogen—which in the third period (III, table 3) comprises 16 per cent of the total nitrogen Upon returning to the original dose of benzoic acid (IV, table 3) the hippuric nitrogen remains at the same level, 16 per cent of the total But when, in addition to the benzoic acid, the benzoyl-leucine is given in such amounts that the benzoyl radical is equivalent to 0 5 gram of benzoic acid, the hippuric nitrogen rises and comprises 37 per cent of the total urinary nitrogen In other words, an increase of 21 per cent is noted in this period (V, table 3) over the corresponding control period (III, table 3) Of this amount the leucine could furnish nearly 11 per cent or half of the extra hippuric nitrogen

Condensed Table 3 Daily average for each period

PERIOD	BENZOIC ACID	BENZOYL-LEUCINE		URINE			
				Total N	Hippuric N	Hippuric N	Extra N
	grams	*gram*	*gram*	*gram*	*gram*	*per cent*	*gram*
I	0			0 565	0 017	3	
II	2 0			0 588	0 104	18	
III	2 5			0 604	0 096	16	
IV	2 0			0 625	0 099	16	
V	2 0	0 415+	0 550	0 640	0 240	37	
VI	2 0			0 738	0 125	17	0 048
VII	0			0 544	0 016	3	

On the basis of the preceding experiment (table 2) the other half of the extra hippuric nitrogen eliminated is probably due to the independent action of the benzoyl radical These results therefore indicate that benzoyl-leucine possesses a double efficiency in the production of hippuric acid first by the conversion of leucine into glycocoll and secondly by the independent action of the benzoyl radical in coupling up available glycocoll

In view of the fact that free leucine does not yield glycocoll, whereas benzoylized leucine evidently does, it seems probable that the leucine does not occur as an intermediary body in the normal metabolism In the third series of experiments an attempt is made to ascertain whether, under conditions in which leucine occurs in the native state as an intermediary product of metabolism, the formation of glycocoll is influenced in any way

With a view to this end, a study of the production of hippuric acid in animals poisoned with phosphorus was made

Condensed Table 4 Daily average for each period Control

PERIOD	BENZOIC ACID	PHOSPHORUS OIL, 1 PER CENT	URINE Total N	Hippuric N	Ammonia N	Extra N
	gram	*cc*	*gram*	*gram*	*gram*	*gram*
I	0	0	0 709	0 012	0 030	
II	0	0 9	0 837	0 016	0 029	0 127

Without the administration of benzoic acid, poisoning with phosphorus does not increase the hippuric acid output Traces of leucine however are found in the urine

Condensed Table 5 Daily average for each period

PERIOD	BENZOIC ACID	PHOSPHORUS OIL 1 PER CENT	URINE Total N	Hippuric N	Ammonia N	Extra N
	grams	*cc*	*gram*	*gram*	*gram*	*gram*
I	0	0	0 509	0 022	0	
II	2 087	0	0 754	0 092	0 027	0 245
III	2 087	0 6	0 882	0 092	0 030	0 373
IV	2 087	1 2	1 292	0 096	0 030	0 783

Condensed Table 6 Daily average for each period

PERIOD	BENZOIC ACID	PHOSPHORUS OIL, 1 PER CENT	URINE Total N	Hippuric N	Ammonia N	Extra N
	grams	*cc*	*gram*	*gram*	*gram*	*gram*
I	0	0	0 815	0 035	0 032	
II	2 5	0	0 999	0 151	0 030	0 184
III	2 5	0 6	1 038	0 113	0 036	0 223

Phosphorus poisoning in a feeding animal, receiving benzoic acid, causes no change in the amount of hippuric acid (and hence glycocoll) eliminated, although it causes a very striking increase in the total nitrogenous metabolism Leucine could not be detected in the urine This result is constant, as repeated experi-

ments show (tables 5 and 6) The hippuric acid output in the poisoned animal can fall below that of the control period, whereas the total urinary nitrogen may rise very high (table 6)

The result is somewhat different when a similar experiment is performed on a fasting animal

Condensed Table 7 Daily average for each period

PERIOD	STATUS	BENZOIC ACID	PHOSPHORUS OIL 1 PER CENT	URINE		
				Total N	Hippuric N	Extra N
		grams	*cc*	*grams*	*gram*	*gram*
I	Feeding	0	0	0 681	0 013	
II	Feeding	2 0	0	0 984	0 083	0 233
III	Fasting	2 0	0	1 103	0 072	0 462
IV	Feeding	2 0	0	0 880	0 118	0 094
V	Fasting	2 0	0 9	1 422	0 123	0 631

The influence of the three factors, namely, fasting, benzoic acid and phosphorus (singly and collectively), upon the course of protein metabolism and glycocoll formation, is clearly illustrated in this experiment (table 7) In the fasting period the production of hippuric acid caused by the benzoic acid is less than in the normal control period, although the total nitrogen output is high Upon returning to normal feeding the total nitrogen drops below and the hippuric nitrogen rises considerably above the preceding period With the development of phosphorus poisoning (in a fasting animal, receiving benzoic acid) and its attendant massive protein decomposition, there occurs an even greater output of urinary nitrogen than in any of the preceding periods, and the hippuric nitrogen also rises above the control fasting period The urine shows the presence of traces of leucine

The increased elimination of hippuric acid, observed in the experiment just described, must be attributed to the excessive breakdown of protein, additional glycocoll probably being derived from that source The very large amount of extra nitrogen eliminated is an evidence of this The absence of any very appreciable amount of leucine may be interpreted in one of two ways viz , that the leucine leads to the production of glycocoll on the

one hand, or, that it leads to the formation of nitrogenous bodies other than glycocoll From the evidence furnished by these experiments it cannot be said, however, that native leucine plays a direct rôle in the production of glycocoll in the body

SUMMARY

Free leucine does not yield glycocoll although it undergoes decomposition in the body

When benzoyl-leucine is given, in addition to a definite dose of benzoic acid, the output of hippuric acid is much greater than the leucine, alone, allows It seems that the increase in the total glycocoll eliminated is partly due to the leucine and partly to the independent action of the benzoyl radical

Phosphorus poisoning in a normal animal causes no increased production of glycocoll or hippuric acid Phosphorus poisoning in an animal receiving benzoic acid also fails to increase the hippuric acid output The fasting animal, when treated with benzoic acid and then poisoned with phosphorus, eliminates more glycocoll than the non-fasting animal, but the increase must be attributed to a greater amount of the substance being available for synthesis with benzoic acid as a result of a massive decomposition of protein

In conclusion it may be said that the experiments here recorded do not afford any definite evidence of the conversion of native leucine into glycocoll In the absence of such evidence it appears likely that much of the glycocoll liberated upon feeding benzoic acid is the result of a synthetic process in the body

The detailed results of the foregoing experiments are given in the accompanying tables

TABLE 1

Rabbit 3 Leucine experiment

DATE		PERIOD	WEIGHT	FOOD (CARROTS)	BENZOIC ACID	LEUCINE N	TOTAL N	TOTAL N DAILY AVERAGE	HIPPURIC N	HIPPURIC N DAILY AVERAGE
			grams	*grams*	*grams*	*gram*	*gram*	*gram*	*gram*	*gram*
Oct	28	I	2000	300			0 169			
	29		1950	300			0 127			
	30		1970	300			0 399	0 128		0 018
	31		1970	300			0 106			
Nov	1	II	1950	300	2 087		0 455		0 177	
	2		1900	300	2 087		0 791		0 128	
	3		1950	300	2 087		0 651		0 152	
	4		1930	300	2 087		0 616		0 105	
	5		2000	300	2 087		0 696		0 111	
	6		1990	300	2 087		0 819	0 705	0 170	0 135
	7		1990	300	2 087		0 707		0 130	
	8		1990	300	2 087		0 991		0 140	
	9		2000	300	2 087		0 665		0 114	
	10		1930	300	2 087		0 651		0 124	
	11	III	2000	300	2 087	0 087	0 959		0 110	
	12		2000	300	2 087	0 106	0 735	0 781	0 136	0 118
	13		1930	300	2 087	0 106	0 658		0 108	
	14	IV	1870	300	2 087		0 735		0 046	
	15		1850	300	2 087		0 875		0 058	
	16		1820	300	2 087		0 721	0 711	0 094	0 088
	17		1870	300	2 087		0 609		0 112	
	18		1850	300	2 087		0 644		0 114	
	19		1870	300	2 087		0 686		0 105	
	20	V	1780	300			0 547		0 031	
	21		1780	300			0 528	0 524	0 015	0 020
	22		1790	300			0 508		0 014	

TABLE 2

Rabbit 7 Ben oyl leucine experiment

DATE		PERIOD	WEIGHT	FOOD (CARROTS)	BENZOIC ACID	BENZOYL	LEUCINE	TOTAL N	HIPPURIC N DAILY AVERAGE	TOTAL N DAILY AVERAGE
				grams	*grams*	*gram*	*gram*	*grams*	*gram*	*gram*
Feb	24	I	Records lost	300				0 507		
	25			300	0			0 532	0 012	0 548
	26			300				0 516		
	27	II		300	2 5			0 749		
	28			300	2 5			0 707	0 085	0 730
	29			300	2 5			0 735		
March	1	III		300	2 5			1 575		
	2			300	2 5			0 791	0 132	0 902
	3			300	2 5			0 861		
	4			300	2 5			0 480		
	5	IV		300	3 125			0 476		
	6			300	3 125			1 260	0 198	0 922
	7			300	3 125			1 029		
	8	V		300	2 5			0 685		
	9			300	2 5			0 952		
	10			300	2 5			0 889		
	11			300	2 5			0 553		
	12			300	2 5			1 113	0 057	0 863
	13			300	2 5			0 571		
	14			300	2 5			1 113		
	15			300	2 5			1 050		
	16	VI		300	*2 5+	0 519	0 088	0 808	0 231	0 868
	17	VII		300	2 5			1 112	0 189	1 442

*Equivalent to 3 1 5 grams benzoic acid

TABLE 3

Rabbit 10 Benzoyl leucine experiment

DATE		PERIOD	WEIGHT	FOOD (CARROTS)	BENZOIC ACID	BENZOYL	LEUCINE	TOTAL N	TOTAL N DAILY AVERAGE	HIPPURIC N DAILY AVERAGE
				grams	*grams*	*gram*	*gram*	*grams*	*gram*	*gram*
March	21		Records lost	300				1 353		
	22			300				0 399		
	23	I		300	0			0 539		
	24			300	0			0 539	0 565	0 017
	25			300	0			0 616		
	26	II		300	2 000			0 420		
	27			300	2 000			0 672	0 538	0 104
	28			300	2 000			0 567		
	29			300	2 000			0 693		
	30	III		300	2 500			0 525		
	30			300	2 500			0 609	0 604	0 096
April	1			300	2 500			0 679		
	2	IV		300	2 000			0 686		
	3			300	2 000			0 448	0 625	0 099
	4			300	2 000			0 742		
	5	V		300	*2 000+	0 415	0 550	0 672	0 640	0 240
	6			300	2 000	0 415	0 550	0 609		
	7	VI		300	2 000			0 756		
	8			300	2 000			0 651	0 738	0 125
	9			300	2 000			0 798		
	10	VII		300	0			0 616		
	11			300	0			0 456	0 544	0 016
	12			300	0			0 560		

Equivalent to 2.500 grams benzoic acid

TABLE 4

Rabbit 2 Phosphorus poisoning control experiment

DATE		PERIOD	WEIGHT	FOOD (CARROTS)	BENZOIC ACID	PHOSPHORUS OIL, 1 PER CENT	TOTAL N	AMMONIA N	TOTAL N DAILY AVERAGE	HIPPURIC N DAILY AVERAGE
			grams	*grams*	*grams*	*cc*	*gram*	*gram*	*gram*	*gram*
Dec	8			100			0 671	0 021		
	9	I					0 672	0 031	0 709	0 012
	10						0 728	0 00		
	11		1910				0 727	0 028		
	12	II	1070			0 6	0 630	0 029		
	13		1930			0 6	0 896	0 029	0 837	0 016
	14		1870			1 2	0 914	0 028		
	*15		1770	230		1 2	0 890	0 031		

*Traces of leucine found in urine

TABLE 5

Rabbit 3 Phosphorus poisoning experiment

DATE	PERIOD	WEIGHT	FOOD (CARROTS)	BENZOIC ACID	PHOSPHORUS OIL 1 PER CENT	TOTAL N	AMMONIA N	TOTAL N DAILY AVERAGE	HIPPURIC N DAILY AVERAGE	HIPPURIC N DAILY AVERAGE
		grams	*grams*	*grams*	*cc*	*grams*	*gram*	*grams*	*gram*	*gram*
Nov 29	I	1830	300			0 462				
30		1810	300			0 518		0 509	0 022	
Dec 1		1860	300			0 546				
2	II	1850	300	2 087		0 700				
3		1850	300	2 087		0 672			0 081	0 081
4		1810	300	2 087		0 756				
5		1860	300	2 087		0 784	0 028		0 078	
6		1800	300	2 087		0 784	0 028	0 754	0 089	
7		1830	300	2 087		0 728	0 034		0 088	
8		1820	300	2 087		0 560	0 025		0 093	0 092
9		1800	300	2 087		0 714	0 025		0 090	
10		1800	300	2 087		0 914	0 017		0 111	
11		1770	300	2 087		0 924	0 029		0 093	
12	III	1770	300	2 087	0 6	0 882	0 022		0 109	0 092
13		1780	300	2 087	0 6	0 882	0 029	0 882	0 086	
14	*IV	1730	300	2 087	1 2	1 358	0 029	1 292	0 126	0 096
15	*V	1700	160	2 087	1 2	1 226	0 041		0 067	

Leucine found in the urine of phosphorus period

TABLE 6

Rabbit 4 Phosphorus poisoning experiment

DATE		PERIOD	WEIGHT	FOOD	BENZOIC ACID	PHOSPHORUS OIL 1 PER CENT	TOTAL N	TOTAL N DAILY AVERAGE	HIPPURIC N DAILY AVERAGE
			grams	*grams*	*grams*	*cc*	*grams*	*grams*	*gram*
January	12	I	2650	300			0 861		
	13		2570	300			0 903	0 815	0 035
	14		2580	300			0 756		
	15		2580	300			0 742		
	16	II	2520	300	2 5		0 770		
	17		2510	300	2 5		1 274	0 999	0 151
	18		2460	200	2 5		1 253		
	19		2440	240	2 5		0 700		
	20	III	2470	200	2 5	0 6	1 253		
	21		2450	200	2 5	0 6	1 155	1 038	0 113
	22		2220	120	2 5	0 6	0 770		
	23		2220	90	2 5	0 6	0 973		
	24*								

Animal died

TABLE 7

Rabbit 5 Phosphorus poisoning experiments

DATE	PERIOD	WEIGHT	FOOD (CARROTS)	BENZOIC ACID	PHOSPHORUS OIL 1 PER CENT	TOTAL N	TOTAL N DAILY AVERAGE	HIPPURIC N	DAILY EXTRA N
		grams	*grams*	*grams*	*cc*	*grams*	*grams*	*gram*	*grams*
Dec 28		2220	300						
29		2260	300						
30	I	2260	300			0 630			
31		2260	300			0 890	0 681	0 013	
Jan 1		2180	300			0 584			
2		2200	300			0 616			
3	II	2200	300	2 000		1 036			
4		2200	300	2 000		0 896			
5		2210	300	2 000		0 644	0 984	0 083	0 230
6		2200	300	2 000		0 644			
7		2200	300	2 000		1 701			
8	III	2065	Fasting	2 000		1 025			
9		2045	Fasting	2 000		1 025	1 103	0 072	0 463
10		2040	Fasting	2 000		1 025			
11		1940	Fasting	2 000		1 337			
12	IV	1960	300	2 000		0 952			
13		2100	300	2 000		0 868			
14		2110	300	2 000			0 880	0 118	0 096
15		2100	300	2 000		0 882			
16		2100	300	2 000		0 819			
17	V	1950	Fasting	2 000	0 9	1 155			
18		1880	Fasting	2 000	0 9	0 730	1 422	0 123	0 631
18 to 19			Fasting	2 000	0 6	1 435			
56 hours	Leucine found in urine								

COMPARATIVE EFFICIENCY FOR GROWTH OF THE TOTAL NITROGEN FROM ALFALFA HAY AND CORN GRAIN[1]

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(Received for publication, September 10, 1912)

It is well known that alfalfa hay, as well as other hays and root crops, contains a considerable part of its nitrogen in non-protein form In alfalfa hay this may amount to 25 per cent of the total nitrogen, as determined by Stutzer's reagent or the tannic acid method Just what the nature of this nitrogen is has not been determined, but it is usually assumed to be a mixture of acid-amides and amino-acids and is generally described under the term "amides" Attempts in this laboratory to isolate individual amino-acids from the water extract of alfalfa hay have failed, but further study of this extract, with the use of the Van Slyke nitrous acid method, shows the presence of free amino groups, but by no means is all of the "amide nitrogen" in the form of simple amides or amino-acids A large proportion is still more complex than these and yet non-precipitable by such reagents as basic lead acetate, tannic acid, etc (The results of a detailed chemical study in this laboratory of the water extract of alfalfa hay will be published shortly)

There is a difference of opinion as to the real worth of "amide nitrogen" for either maintenance, growth or milk production Armsby[3] in his latest tables of feeding standards, excludes it entirely

[1] Published by permission of the Director of the Agricultural Experiment Station

[2] Credit is due Mr P H Wessels for part of the analytical work reported in this investigation

[3] Farmers Bulletin 346, U S Department of Agriculture

from his calculations of the available nitrogen. On the other hand, Henry[4] and Jordan,[5] at least in their tables of the composition of feeds, give it full value. Nor has scientific inquiry as to its worth been wanting. The whole question is to some extent bound up with the question of the nature and extent of protein hydrolysis in animal digestion. It appears from the work of Cohnheim,[6] Kutscher and Seemann[7] and Abderhalden, Baumann and London[8] that digestion proceeds to the formation of abiuret products in the form of polypeptides and the comparatively simple monoamino- and diamino-acids. Feeding experiments with predigested protein have also thrown light on this question. Loewy[9] showed that protein, digested until the biuret reaction was absent, was capable not only of maintaining life, but of maintaining nitrogen equilibrium and even of leading to nitrogen retention. Henderson and Dean[10] used the products of acid hydrolysis of proteins. They also record nitrogen retention. Others, as Henriques and Hanson,[11] were unable to get as good results with the products of acid hydrolysis as when the hydrolysis was carried on by either pepsin or trypsin, although they showed that the products from acid digestion were excellent protein sparers. Numerous experiments in this direction have been carried on by Abderhalden and his pupils.[12] He has worked with both mice and dogs. His earlier experiments tended to show that a protein digested with an enzyme until the biuret reaction was absent could take the place of protein in the diet, but that the acid hydrolytic products could not. Later Abderhalden[13] brought forth evidence to show that nitrogen equilibrium could be maintained and a retention occur when the products of acid hydrolysis alone were used. With an artificial mixture of amino-acids he was also able to secure nitrogen equilib-

[4] *Feeds and Feeding.*

[5] *The Feeding of Animals.*

[6] *Zeitschr. f. physiol. Chem.*, xlix, p. 64, 1906.

[7] *Ibid.*, xxxiv, p. 527, 1901.

[8] *Ibid.*, li, p. 384, 1907.

[9] *Arch. f. exp. Path. u. Pharm.*, xlviii, p. 303, 1902.

[10] *Amer. Journ. of Physiol.*, ix, p. 386, 1903.

[11] *Zeitschr. f. physiol. Chem.*, xlix, p. 113, 1906.

[12] *Ibid.*, xlii, p. 528, 1904.

[13] *Ibid.*, lxxvii, p. 23, 1912.

rium[14] and effect a partial nitrogen retention Abderhalden[15] has even furnished experimental evidence, although not very convincing, that with a diet rich in carbohydrates and fat, he was able to secure nitrogen retention when the sole source of nitrogen in the diet was an ammonium salt This work was done with dogs

From the above experimental work it is apparent that the simple amino-acids have direct nutritive value and very probably represent a large proportion of the normal end products of protein digestion The work of Bergmann,[16] Howell[17] and Folin and Denis[18] would indicate that they are absorbed into the blood and used directly for cell repair and protein synthesis

The fact that our hays and root crops may contain a certain proportion of nitrogen in true amide form, as in asparagine or glutamine, raises the question of the nutritive value of their nitrogen The part of it which is in the α-amino-position must possess that value which can be assigned to amino-acid nitrogen As early as 1894, Weiske[19] showed that for herbivora asparagine was a very effective protein sparer Zuntz[20] had suggested that the "amide nitrogen" is built into protein by the aid of intestinal bacteria before it becomes utilizable by these animals This view was also shared by Muller,[21] who has shown that the intestinal bacteria could form protein from asparagine and also from ammonium tartrate Schulze[22] was also of the opinion that this is the way in which the amides are useful to herbivora On the other hand Morgen,[23] working with sheep, has shown that there was not a building of indigestible protein by the intestinal bacteria when ammonium acetate and asparagine were fed This view does not prove or disprove the possibility of the formation of bacterial proteins as intermediary bodies in the utilization of ammonium

[14] *Zeitschr f physiol Chem*, lxxvii, p 23, 1912
[15] *Ibid*, lxxviii, p 1, 1912
[16] *Beitr z chem Physiol u Path*, vi, p 40, 1905
[17] *Amer Journ of Physiol*, xvii, p 273, 1906
[18] This *Journal*, xi, p 87, 1912
[19] *Zeitschr f Biol*, xxx, p 254, 1894
[20] *Arch f d ges Physiol*, xlix, p 477, 1891
[21] *Ibid*, cxii, p 245, 1906
[22] *Zeitschr f physiol Chem*, lvii, p 67, 1908
[23] *Landw Versuchsstat*, lxxiii, p 283, 1912

salts and asparagine by herbivora Morgen and his associates have shown further that the non-protein nitrogen, as in ammonium salts or asparagine, besides being capable of supporting maintenance, could also contribute to the production of milk proteins Kellner,[24] experimenting with lambs, concluded that asparagine and ammonium salts were capable of aiding in the maintenance of nitrogen equilibrium, but were incapable of conversion into body protein

The evidence from the literature cited above appears to show that with herbivora the "amides," or non-protein nitrogen, can serve to maintain the protein tissues of the body and there is certain evidence that they can also support milk production Evidence is lacking that growth can be produced

With rats Politis[25] was unable to secure any evidence of protein sparing by asparagine when the latter was the sole source of nitrogen The same result was obtained by Henriques and Hanson[26] but, when the latter used the expressed juice of etiolated beans or peas, they found that a sparing action was partly effected The "amide nitrogen" from potatoes or roots was ineffective as a protein sparer when fed to rats Luthje[27] showed the same thing to be true with rabbits when the sole source of nitrogen was the protein-free "amide" material obtained from fresh potatoes Voltz[28] concluded that herbivora can make use of "amide nitrogen" and the nitrogen in ammonium salts and he has lately[29] reached the same conclusion for carnivora

Evidently the kind of animal is an important matter in determining how effective these simple nitrogenous substances may be for both maintenance and growth but this is probably more a matter of individual temperament and influence on appetite than of an actual difference in the nutrition processes under normal conditions In the literature the idea prevails that herbivora can apparently make more efficient use of these simple nitrog-

[24] *Arch f d ges Physiol*, cxiii, p 480, 1906, *Zeitschr f Biol*, xxxix, p 313, 1900

[25] *Zeitschr f Biol*, xxviii, p 492, 1891

[26] *Zeitschr f physiol Chem*, liv, p 169, 1907

[27] *Arch f d ges Physiol*, cxiii, p 547, 1906

[28] *Ibid*, cxvii, p 541, 1907

[29] *Zeitschr f physiol Chem*, lxxix, p 415, 1912

enous substances than the omnivora or carnivora Yet Abderhalden claims to have caused nitrogen retention in a dog with an artificial mixture of amino-acids and even with ammonium salts One trouble in the feeding of concentrated plant extracts is that they usually lead to intestinal derangement and diarrhea follows This may be expected from the large salt content of such concentrates

Plan of experiment

The plan of our experiment was to secure experimental data on the rate of nitrogen retention by growing heifers when the source of the nitrogen in the ration was mainly either the corn grain or the whole alfalfa plant The corn kernel contains only traces of "amide nitrogen" and would serve as an excellent check on the alfalfa periods The corn ration consisted of corn meal, gluten feed and corn stover The gluten feed was added for the purpose of bringing the nitrogen content of the ration up to a good growing level It is necessary with this class of animals to use some roughage and, as the corn stover introduced very little available nitrogen and at the same time would maintain an alkaline urine, it was used The alfalfa ration consisted mainly of alfalfa hay and corn starch Part of the alfalfa was ground in order that the bulk of the material might be reduced and at the same time a small amount of corn stover was introduced It was believed that the corn stover would help consumption, but later experiments have shown that this is unnecessary The starch was used for the purpose of making the net available energy of the two rations closely comparable It was believed that, should the rate of nitrogen retention on the two rations through a long period of observation be approximately alike, we would have substantial evidence that the "amide nitrogen" was being used for growth We would certainly have evidence whether the total nitrogen from one source and in the proportion used was as efficient for growth as from the other source And for us this is an important point

Two animals were used in the experiment, one receiving the corn ration while the other received the alfalfa ration After six or eight weeks' observation, the rations were changed and the animal originally receiving the corn ration went to the alfalfa

ration and *vice versa* This would obviate any differences in individual powers of growth or capacity for utilization, which is an important factor in such experiments

A record of income and outgo of nitrogen was quantitatively kept, the feces, urine, etc, being quantitatively collected, sampled and analyzed With the ration, salt and water were fed *ad libitum* Each animal was fed such portions of the ration as would be entirely consumed At a definite time of the day, weekly weighings of the animals were made

Experiment in 1910–1911

Two young growing heifers, of approximately 400 to 500 pounds weight, were chosen They were grade Holsteins, number 1 weighing 505 pounds and number 2, 440 pounds The rations fed are shown in table I These figures are taken from Henry's Feeds and Feeding and Armsby's Tables for Production Therms This table simply shows the proportion in which the materials in an air dried condition were mixed and the approximate likeness of the two rations in production therms, nutritive ratio and digestible protein This last figure is, of course, based on the total nitrogen and includes the "amide nitrogen" The table also includes in the last column the actual grams of nitrogen contained in such weights of the feed as are indicated in column 2

It should be noted that the nutritive ratio of the alfalfa ration was 1 9 If the "amide nitrogen" is not included, but only true protein made the basis of the calculation, then the nutritive ratio becomes 1 12 4, a very wide ratio for growth

From table I it will be seen that on the basis of average analyses, the two rations were very nearly alike in total digestible protein and available therms From actual nitrogen determinations, the total nitrogen intake in the two rations was also very closely alike With the consumption of approximately equal quantities of air dried material, the intake of total digestible nitrogen, as well as total nitrogen, was substantially identical in both rations We had no difficulty in securing a liberal consumption of the rations and approximately equal portions by either animal The daily consumption for the entire sixteen weeks varied from 7 to 10 pounds

TABLE I

Composition of materials used

	POUNDS	DIGESTIBLE NUTRIENTS			PRODUCTION THERMS	NUTRITIVE RATIO	TOTAL N
		Protein	Carbohydrate	Fat			
		pounds	*pounds*	*pounds*			*grams*
Corn meal	5	0 40	3 33	0 22	4 44		32 46
Gluten feed	2	0 41	0 97	0 17	1 58	1 8 2	39 04
Corn stover	7	0 12	2 26	0 05	1 85		34 95
Total	14	0 93	6 56	0 44	7 87		106 45
Corn stover	3	0 05	0 97	0 02	0 79		14 98
Alfalfa hay	5	0 88	3 16	0 09	2 75	1 9 0	56 97
Alfalfa meal	3						34 21
Starch	4 2	0 00	4 00	0 00	4 20		1 52
Total	15 2	0 93	8 13	0 11	7 74		107 68

In all, sixteen weeks of actual observation are involved in this first experiment, 1910–1911 Before the records were begun there was a preliminary period of ten days' feeding In period I, of eight weeks, animal 1 received the corn ration and animal 2 the alfalfa ration At the end of that period the rations were changed This was done slowly and by substituting a pound of one ration for a pound of the other, consuming in all eight days for the transfer In period II, animal 1 received the alfalfa ration, while animal 2 received the corn ration In tables II and III are included the records of this first experiment The data are condensed from daily records to weekly composites and represent in grams the nitrogen consumed per week and the nitrogen excreted and retained In addition are columns showing the pounds of feed consumed and a record of the gain in live weight

Animal 1 stored 451 7 grams of nitrogen during the corn period or 13 4 per cent of the total intake and 754 2 grams on the alfalfa ration or 20 4 per cent of the total intake It will be noted that there is considerable oscillation in the weekly records of the amounts of nitrogen retained This is illustrated in charts I and II, which are graphic records of nitrogen retention and nitrogen consumption It is very doubtful if this means a variation in the rates of cell growth and nitrogen storage but, rather, a

TABLE II

Record of balance of income, outgo and retention of nitrogen, feed consumed and gain in live weight

Animal 1 Period 1 Corn ration

DATE 1910–1911	FEED	N INTAKE	N IN FECES	N IN URINE	N RETAINED	LIVE WEIGHT (INITIAL 505 LBS)
	pounds	*grams*	*grams*	*grams*	*grams*	*pounds*
Dec 2–8	8	425 8	182 4	186 9	55 5	513
9–15	7–8	418 2	199 2	200 0	19 0	521
16–22	7	380 9	168 6	178 5	33 8	493
23–29	8–9	430 7	175 3	173 0	82 4	509
Jan 30–5	8	425 8	190 9	161 7	73 2	520
6–12	8	409 9*	185 4	163 1	61 4	524
13–19	9	431 2	222 7	169 0	39 5	531
20–26	9	431 2	204 9	139 4	86 9	534
					451 7	

Animal 1 Period II Alfalfa ration

DATE 1910–1911	FEED	N INTAKE	N IN FECES	N IN URINE	N RETAINED	LIVE WEIGHT (INITIAL 505 LBS)
Feb 4–10	9	427 4	187 6	152 4	87 4	544
11–17	9	427 4	184 0	186 7	56 7	544
18–24	9–10	454 6	187 8	148 4	108 4	556
25–3	10	474 6	208 8	164 2	100 2	576
Mar 4–10	10	474 6	218 6	155 8	100 2	575
11–17	10	474 6	214 9	144 1	115 6	579
18–24	10	474 6	220 0	170 4	84 2	581
25–31	10	474 6	217 7	155 4	101 5	592
					754 2	

* New corn stover used 7 pounds contained 24 33 grams of N

ence in the amounts of nitrogen excreted, as determined by the artificial periods of observation prescribed The efficiency of the total alfalfa nitrogen for growth was for animal 1 somewhat greater than that of the corn kernel While we do not care to lay too much stress on the positive figures for alfalfa obtained in this case, nevertheless, the data show that the total nitrogen from this plant was not inferior for growth to that from the corn kernel In the transition period from the corn to the alfalfa ration, there was no sudden rise in the nitrogen output, either in the feces or urine The ingestion of a large proportion of "amide nitrogen" with the change to the alfalfa ration, should have led to such increased excretion had this not been involved in the normal

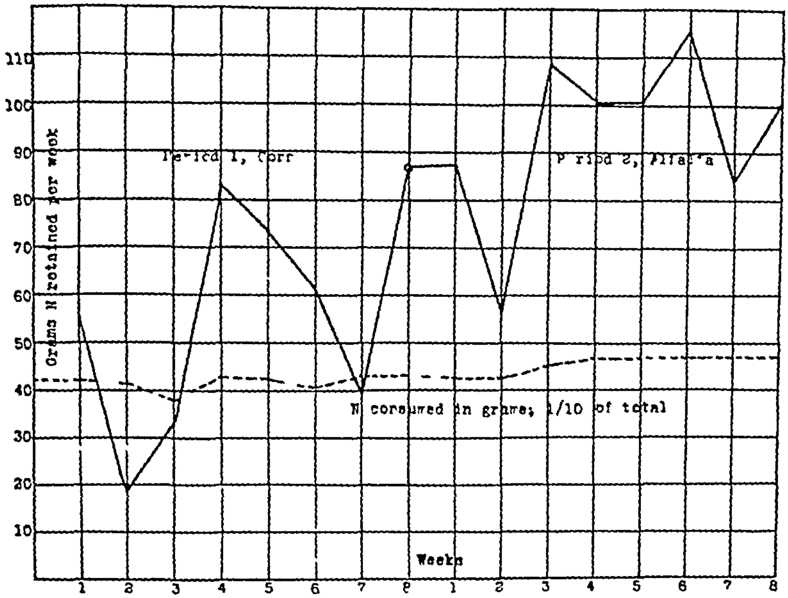

CHART I, ANIMAL 1 CURVE OF N RETENTION (UNBROKEN LINE) CURVE OF N CONSUMPTION (DOTTED LINE)

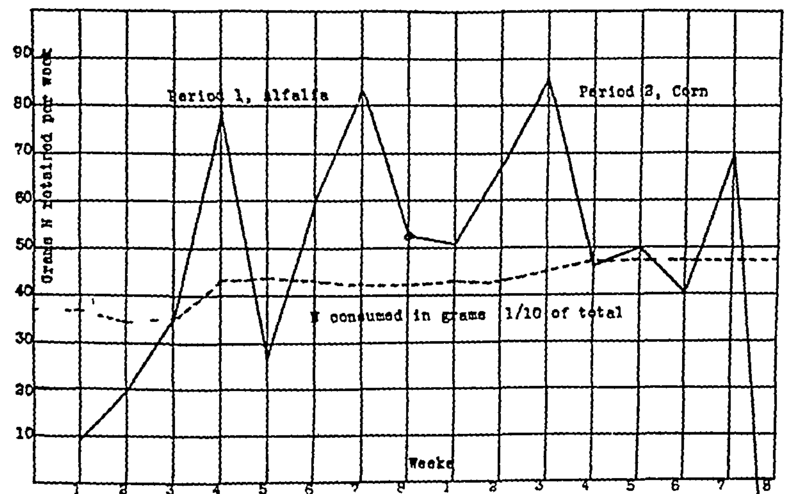

CHART II, ANIMAL 2 CURVE OF N RETENTION (UNBROKEN LINE) CURVE OF N CONSUMPTION (DOTTED LINE)

TABLE III

Record of balance of income, outgo and retention of nitrogen, feed consumed and gain in live weight

Animal 2 Period I Alfalfa ration

DATE 1910–1911	FEED	N INTAKE	N IN FECES	N IN URINE	N RETAINED	LIVE WEIGHT (INITIAL 440 LBS)
	pounds	*grams*	*grams*	*grams*	*grams*	*pounds*
Dec 2–8	7–8	375 4	167 6	198 0	9 8	445
9–15	7	347 1	149 1	178 0	20 0	426
16–22	7–8	354 0	132 5	186 4	35 1	430
23–29	8–9	439 2	181 3	178 4	79 5	447
Jan 30–5	9	446 3	211 0	207 9	27 4	452
6–12	9	430 2*	206 0	163 9	60 3	453
13–19	9	427 5	208 9	134 9	83 7	456
20–26	9	427 5	197 2	177 5	52 8	454
					368 6	

Animal 2 Period II Corn ration

DATE 1910–1911	FEED	N INTAKE	N IN FECES	N IN URINE	N RETAINED	LIVE WEIGHT (INITIAL 440 LBS)
Feb 4–10	9	431 2	191 3	188 5	51 4	480
11–17	9	431 2	203 3	160 2	67 5	481
18–24	9–10	458 6	231 6	141 1	85 9	502
25–3	10	*479 2	239 6	192 8	46 8	508
Mar 4–10	10	479 2	257 4	171 9	49 9	522
11–17	10	479 2	247 0	191 7	40 5	514
18–24	10	479 2	219 1	190 3	69 8	527
25–31	10	479 2	271 6	249 2	−41 4	525
					369 4	

* New corn stover used 7 pounds contained 24 33 grams of N

processes of nitrogen utilization and elimination Of the total nitrogen absorbed, animal 1 retained during the corn period 24 7 per cent and during the alfalfa period 36 9 per cent

Animal 2 stored 368 6 grams of nitrogen during the alfalfa period or 11 3 per cent of the total intake and 369 4 grams on the corn ration or 9 9 per cent The same oscillation in weekly storage is also to be observed with this animal Apparently animal 2 did not possess the same powers of growth as animal 1 In neither period was the amount stored as great as in the case of animal 1 At the end of period II, this animal suffered somewhat from diarrhea, a fact which accounts for the negative balance of that period With this animal the amount of nitrogen retention on

the two rations was practically alike, indicating again no inferiority of the alfalfa nitrogen for processes of growth

Again in this case there was no sudden decrease of nitrogen in the feces or urine as the change from the alfalfa ration to the corn ration was made During the alfalfa period animal 2 stored 20 5 per cent of the absorbed nitrogen and 18 8 per cent during the corn period

Attention should be called to the discrepancy in the live weight gains as compared with actual nitrogen storage Animal 1 gained in gross live weight during the sixteen weeks some 79 pounds, with an actual storage of 1205 9 grams of nitrogen Animal 2 gained 80 pounds in live weight during the same period, but actually stored but 738 grams of nitrogen or approximately three-fifths as much as animal 1

Experiment in 1911–1912

The plans of experimentation in 1911–1912 were substantially those of the previous year Two young growing heifers of grade Holstein breeding and about 300 to 400 pounds in weight were again chosen The rations used were of the same kind of materials as in the previous experiment, except that no corn stover was introduced into the alfalfa ration This ration consisted wholly of alfalfa and starch Again a part of the alfalfa hay was reduced to meal in our own grinders for the purpose of reducing the bulk The composition of the ration is appended in table IV

It will be seen that the rations were very similar in respect to total digestible nutrients, therms and nutritive ratio The total nitrogen intake with like weight of air dried mixture was also substantially indentical

In this experiment there was a preliminary three weeks' feeding period with a low nitrogen ration This ration consisted of 4 pounds of wheat straw, 3 pounds of corn starch, 0 2 pound of cane sugar and 1 ounce of calcium phosphate Salt was given freely This preliminary period was to determine, on as low a nitrogen intake as practicable with the materials available, what the creatinine output would be and the relation of the creatinine nitrogen to the total nitrogen of the urine McCollum[30] has shown, with

[30] Research Bulletin 21, Wisconsin Exp Sta

TABLE IV

Composition of materials used

	Feed	Digestible nutrients			Production therms	Nutritive ratio	Total N
		Protein	Carbohydrate	Fat			
	pounds	*pounds*	*pounds*	*pounds*			*grams*
Corn meal	5	0 40	3 33	0 22			33 36
Gluten feed	2	0 41	0 97	0 17			40 42
Corn stover	7	0 12	2 26	0 05			37 67
Total	14	0 93	6 56	0 44	7 8	1 8 2	111 45
Alfalfa hay	5						60 33
Alfalfa meal	4	0 99	3 51	0 10			51 33
Starch	5		5 00				1 36
Total	14	0 99	8 51	0 10	8 0	1 8 5	113 02

pigs on practically nitrogen-free rations but with the energy requirement satisfied, that the creatinine excretion reaches a maximum and is a fairly definite proportion of the total urinary nitrogen This would indicate that it was a constant end product of endogenous metabolism Further he has shown that with increasing nitrogen retention in pigs there is a fairly definite proportional increase in the creatinine output This would indicate that creatinine would serve as an index of protein storage It was to learn whether such quantitative relationships held for herbivora that we instituted this preliminary feeding period

Effect of low nitrogen intake on creatinine excretion In table V are given the data for this preliminary three weeks' period, with both animals, on the effect of low nitrogen intake on creatinine output This is expressed in grams and the per cent of creatinine nitrogen of the total urinary nitrogen A week of feeding preceded the taking of these records

In animal 3 the proportion of the total nitrogen of the urine as creatinine nitrogen rose as high as McCollum has reported for pigs receiving nitrogen-free rations, and on single days it went as *high* as 25 per cent With animal 4 the ratio did not rise as high, although in the first week it was approximately 20 per cent After the first week it decreased in both cases, possibly due to the losses of body protein incident to the low nitrogen ration The average

TABLE V

Record of low nitrogen feeding and creatinine output

Animal 3

DATE	N INTAKE	N IN FECES	N IN URINE	N LOSS	DAILY AVERAGE CREATININE	CREATININE N AS AVERAGE PERCENTAGE OF TOTAL URINARY N
	grams	*grams*	*grams*	*grams*	*grams*	*per cent*
Dec 12–18	39 48	59 97	43 73	64 2	3 67	22 0
19–25	39 48	63 66	34 11	58 2	2 71	21 0
26–Jan 1	39 48	66 50	36 26	63 2	2 14	15 2
Animal 4						
Dec 12–18	41 09	78 97	47 85	85 7	3 39	18 4
19–25	41 09	79 75	51 88	90 5	2 55	11 9
26–Jan 1	41 09	67 61	33 28	59 8	1 61	12 9

daily grams of creatinine excreted were also highest in the first week in the case of both animals and decreased perceptibly under nitrogen starvation It should be noted that this high ratio of creatinine nitrogen to total urinary nitrogen was reached in the first week of the record and the second week of feeding This high ratio was reached somewhat earlier than was observed by McCollum with pigs on nitrogen-free diets

We shall discuss the creatinine rise on our growing ration further on in this paper

After the preliminary feeding the animals were immediately placed on the growing rations of corn and alfalfa They were to receive these rations for six weeks and then be immediately changed to the ration of the other It is very essential that this should be done as then any individual differences in powers of growth are obviated

The records of nitrogen income, outgo and storage, as well as columns showing the food consumption and increase in live weight, are given in tables VI and VII Nitrogen records are in grams per week and are seven-day composites of the daily record

Animal 3 stored during the alfalfa period 592 2 grams of nitrogen or 28 7 per cent of the total intake During the corn period 615 6 grams of nitrogen were stored or 24 1 per cent of the total nitrogen consumed During the first week of record on the alfalfa

ration 45 per cent of the total nitrogen ingested was stored This very high efficiency is to be attributed to the partial nitrogen depletion the animal had suffered during the preliminary period of partial nitrogen starvation, but even after the first week there was a continued high efficiency of storage, which was maintained on both rations for the entire period of record This is in marked contrast to animals 1 and 2 where the storage was not much over 10 per cent of the total nitrogen ingested Both animals 3 and 4 were somewhat younger and lighter in weight than 1 and 2, which are additional factors in the rate of growth

The data show that the nitrogen in the alfalfa ration under the conditions of the experiment is just as efficient for growth as that of the corn kernel Of the nitrogen absorbed from the intestine during the alfalfa period 48 per cent was retained while during the corn period 40 per cent of the absorbed nitrogen remained in the body The sudden change from the alfalfa ration to the corn

TABLE VI

Record of income, outgo, retention of nitrogen, feed consumed and gain in live weight

Animal 3 Period I Alfalfa ration

DATE	FEED	N INTAKE	N IN FECES	N IN URINE	N RETAINED	LIVE WEIGHT (INITIAL 348 LBS)
	pounds	*grams*	*grams*	*grams*	*grams*	*pounds*
Jan 2–8	6–7	339 0	114 5	71 0	153 5	347
9–15	6–7	339 0	143 3	87 0	108 7	343
16–22	6–7	339 0	133 5	111 6	93 9	348
23–29	6–7	339 0	133 7	119 3	86 0	352
30–Feb 5	6–7	363 2	155 0	122 4	85 6	356
Feb 6–12	6–7	339 0	153 7	120 8	64 5	358
					592 2	

Animal 3 Period II Corn ration

DATE	FEED	N INTAKE	N IN FECES	N IN URINE	N RETAINED	LIVE WEIGHT
Feb 13–19	6–7	358 2	140 4	151 1	66 7	372
20–26	7	413 9	163 6	118 9	131 4	386
27–Mar 4	7–8	445 7	196 0	122 7	127 0	393
Mar 5–11	7–8	445 7	176 5	149 3	119 9	401
12–18	7–8	445 7	170 4	179 2	96 1	404
19–25	7–8	445 7	183 9	187 3	74 5	415
					615 6	

ration should have materially decreased the nitrogen in the urine if it were true that practically one-fourth of the nitrogen of the alfalfa was in useless form and unsuited for building purposes The hypothesis that there is formed in the digestive tract from the "amide nitrogen" indigestible bacterial proteins also does not receive support from our data In the two weeks before and after the change in ration the nitrogen content of the feces is practically identical Apparently the two sources of nitrogen are being handled in identical fashion

Animal 4 stored during the corn period 879 3 grams of nitrogen or 35 4 per cent of the total intake During the alfalfa period 822 8 grams were stored or 29 6 per cent of the total nitrogen ingested Here, again, as with animal number 3, the first week showed a very high nitrogen retention, amounting to 44 per cent of the total intake This followed immediately a period of nitrogen starvation and accounts for such high efficiency This com-

TABLE VII

Record of income, outgo, retention of nitrogen, feed consumed and gain in live weight

Animal 4 Period I Corn ration

DATE	FEED	N INTAKE	N IN FECES	N IN URINE	N RETAINED	LIVE WEIGHT (INITIAL 387 LBS)
	pounds	*grams*	*grams*	*grams*	*grams*	*pounds*
Jan 2–8	6–7	342 2	112 1	77 2	152 9	379
9–15	7	390 0	134 6	131 7	123 7	382
16–22	7	390 0	147 3	118 5	124 2	385
23–29	8	451 9	161 5	103 4	187 0	400
30–Feb 5	8	451 9	173 9	125 5	142 5	407
6–12	8	451 9	166 4	126 5	149 0	415
					879 3	

Animal 4 Period II Alfalfa ration

DATE	FEED	N INTAKE	N IN FECES	N IN URINE	N RETAINED	LIVE WEIGHT
Feb 13–19	7–8	443 8	169 9	113 8	160 1	424
20–26	8–9	476 1	174 1	123 6	178 4	435
27–4	9	492 2	194 6	135 5	162 1	440
Mar 5–11	8	451 9	182 8	185 4	83 7	430
12–18	8	451 9	156 7	171 0	124 2	442
19–25	8	451 9	169 6	168 0	114 3	432
					822 8	

paratively high retention continued during both periods of feeding and was practically parallel with that of animal 3 During the corn period 55 per cent of the total nitrogen absorbed from the intestine was retained, while in the alfalfa period 48 per cent was retained With both animals there was a slow decrease in the rate of nitrogen retention as the periods progressed When the rations were suddenly changed there was in both cases an increase in the rate of nitrogen retention for a two weeks' period and then a decrease Such variations are difficult to explain and at present can only be interpreted on the basis of lags in elimination Such rises and falls may occur any time in the period of feeding as illustrated in charts III and IV, which show the curves of nitrogen

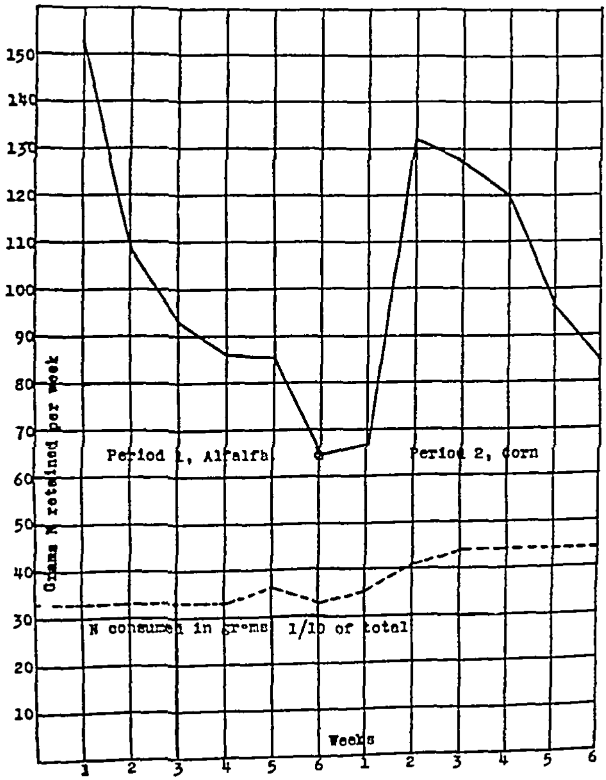

CHART III, ANIMAL 3 CURVE OF N RETENTION (UNBROKEN LINE) CURVE OF N CONSUMPTION (DOTTED LINE)

..ation and consumption for animals 3 and 4 Animal 4 gives clear-cut demonstration of the failure of a sudden change from he corn to the alfalfa ration to raise the nitrogen excretion in ther urine or feces Even for two weeks, which is beyond the .. of a lag of elimination, the nitrogen in the feces and urine l not rise perceptibly above what it was in the last week on he corn ration

At the end of this period of feeding, both animals were again laced upon the nitrogen-low ration of straw and starch for a eriod of ten days for the purpose of again determining the ratio f creatinine nitrogen to the total urinary nitrogen In the case

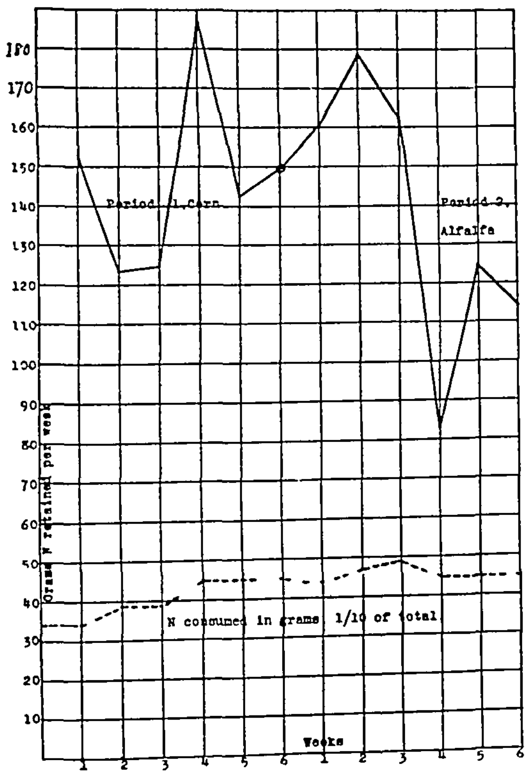

Chart IV, animal 4 Curve of N retention (unbroken line) Curve of N consumption (dotted line)

and of Ringer and Lusk[2] has shown that glycocoll and *i*-alanine are completely converted into dextrose by the phlorhizinized dog, and that three atoms of carbon contained in aspartic and glutamic acids are also convertible into dextrose These four amino-acids yield about one-half of the sugar derived from protein metabolism in phlorhizin glycosuria [3] This information presented the possibility of testing the validity of Rubner's hypothesis, and as far back as 1908 the writer[4] gave 40 grams of dextrose to a dog and then 20 grams of dextrose to which 16 grams of alanine were added, and noted no change in the metabolism during twenty-four hours as determined by the Pettenkofer-Voit respiration apparatus Other similar experiments showed that the addition of 24 grams of tyrosine and of 40 grams of glutamic acid to a standard diet were without effect upon the metabolism of the animal These experiments have never been published in detail and there is now no reason for such publication As will be shown in paper six, the action of the amino-acids was masked by the mass action of dextrose or of the standard diet given

In paper three, it was demonstrated that 20 grams of dextrose administered to Dog II caused in one instance no effect on metabolism, while in another instance it caused a slight increase and this only during the second hour If 25 grams of glycocoll or 20 grams of alanine which are completely convertible into 20 grams of dextrose in metabolism, behave like dextrose, then, after their ingestion, they should cause only a slight rise in the heat production If glutamic acid be given it might show a "specific dynamic" action, for three of its carbon atoms are convertible into dextrose while two on oxidation might furnish free heat to the body Also tyrosine, with its many cleavages before it reaches a stage for use by the cells in metabolism, might show a pronounced effect upon the heat production The writer freely admits that these were the results which he was prepared to find Prior to the work here presented, his ideas on this subject had been essentially derived from the teachings of Rubner

[2] Ringer and Lusk *Zeitschr f physiol Chem*, lxvi, p 106, 1910
[3] Lusk Phlorhizinglukosurie, *Ergeb d Physiol*, xii, p 315, 1912
[4] Lusk *Proc Soc Exp Biol and Med*, vii, p 136, 1910

II EXPERIMENTAL PROCEDURE

The experiments were conducted as in paper three upon Dog II The same standard diet was administered at six o'clock in the evening The amino-acid was dissolved or suspended in 150 cc of warm water containing 2 grams of Liebig's extract of beef and given at noon

The total nitrogen and urea plus ammonia nitrogen were determined in hourly periods in the dog on occasions when the meat extract was given, and the animal was not put in the calorimeter Under similar conditions, total nitrogen and urea plus ammonia nitrogen were determined in hourly periods after an amino-acid had been ingested with the Liebig broth The difference between the urea plus ammonia nitrogens in the two instances was assumed to represent the amino-acid destruction of the hour The difference between the total nitrogens of corresponding hours represented approximately the amino-acid metabolized plus the amino-acid eliminated as such The true protein metabolism of the time was assumed to be the nitrogen which would have been eliminated had no amino-acid or beef extract been administered From the values thus calculated, approximate figures were obtained which represented the protein and amino-acid metabolism during the hours when the animal was in the calorimeter A similar procedure was employed with Dog I in paper two when meat was ingested

In order to determine the indirect calorimetry when amino-acids are metabolized, the table on page 158 was prepared

III EXPERIMENTAL PART

The full analytical details of all the experiments are given in the Appendix, Table I

A Liebig's extract of beef

Both Rubner[5] and Burgi[6] have demonstrated that the constituents of Liebig's extract of beef are very largely ready for elimination in the urine The researches of Pawlow and his pupils

[5] Rubner *Zeitschr f Biol*, xix, p 343, 1883

[6] Burgi *Archiv für Hygiene*, li, p 1, 1904

Physiological values of amino-acids when oxidized in the organism, calculated by deducting the value of urea formed from them in metabolism

	Formula	Molecular weight	Per cent N	Calories: Actual per gram	Calories: Physiological per gram	Calories: Physiological per gram N	In oxidation of 1 gram of substance: O_2	In oxidation of 1 gram of substance: CO_2	In oxidation of 1 gram of substance: O_2	In oxidation of 1 gram of substance: CO_2	R Q	Caloric value 1 liter O_2	1 gram N = O_2	1 gram N = CO_2	1 gram N = C in resp
							grams	*grams*	*liters*	*liters*			*grams*	*grams*	*grams*
Glycocoll	$C_2H_5NO_2$	75	18 66	*3 110	1 846	9 89	0 64	0 88	0 448	0 448	1 00	4 12	3 43	4 71	1 28
Alanine	$C_3H_7NO_2$	89	15 74	*4 401	3 037	19 30	1 08	1 236	0 756	0 629	0 83	4 15	6 86	7 85	2 14
Glutamic acid	$C_5H_9NO_4$	147	9 52	*3 662	3 146	33 05	0 98	1 347	0 686	0 686	1 00	4 593	10 32	14 18	3 87
Leucine	$C_6H_{13}NO_2$	131	10 68	†6 525	5 946	55 67	1 832	1 840	1 282	0 938	0 73	4 638	17 15	17 23	4 70
Tyrosine	$C_9H_{11}NO_3$	181	7 73	*5 915	5 496	71 10	1 679	2 066	1 175	1 051	0 89	4 678	21 72	26 73	7 29

* Emery and Benedict *Amer Journ of Physiol* xxviii p 307 1911 Urea yields 2 528 calories according to these authors which is essentially the same as 2 523, correctly established by Rubner in 1885

† Stohmann and Langbein *Journ f prakt Chem*, xliv p 383 1891

Physiological values of amino-acids when oxidized in the organism, calculated by deducting the value of urea formed from them in metabolism

	FORMULA	MOLECULAR WEIGHT	PER CENT N	CALORIES			IN OXIDATION OF 1 GRAM OF SUBSTANCE					CALORIC VALUE 1 LITER O_2	1 GRAM N =		
				Actual	Physiological										
				per gram	per gram	per gram N	O_2	CO_2	O_2	CO_2	R Q		O_2	CO_2	C in resp
							grams	*grams*	*liters*	*liters*			*grams*	*grams*	*grams*
Glycocoll	$C_2H_5NO_2$	75	18 66	*3 110	1 816	9 89	0 64	0 88	0 418	0 448	1 00	4 12	3 13	4 71	1 28
Alanine	$C_3H_7NO_2$	89	15 74	*4 401	3 037	19 30	1 08	1 236	0 756	0 629	0 83	4 15	6 86	7 85	2 14
Glutamic acid	$C_5H_9NO_4$	147	9 52	*3 662	3 146	33 05	0 98	1 347	0 686	0 686	1 00	4 593	10 32	14 18	3 87
Leucine	$C_6H_{13}NO_2$	131	10 68	†6 525	5 916	55 67	1 832	1 840	1 282	0 938	0 73	4 638	17 15	17 23	4 70
Tyrosine	$C_9H_{11}NO_3$	181	7 73	*5 915	5 496	71 10	1 679	2 066	1 175	1 054	0 89	4 678	21 72	26 73	7 29

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of 0 132 per hour, then 0 549 gram would be eliminated during four hours and ten minutes

Considering now the elimination of nitrogen during the same interval of time after meat extract containing 0 156 gram of nitrogen was administered, the following differences are found

Date	Total N	Extra N
February 6	0 723 − 0 549 =	0 174
March 22	0 747 − 0 549 =	0 198
March 28	0 672 − 0 549 =	0 123

It is, therefore, evident that the nitrogen ingested in the meat extract is essentially eliminated during the first four hours after its administration

To estimate the protein metabolism of February 6, when meat extract was given, one might assume (1) the value of the hourly nitrogen elimination of the day previous (= 0 094), or (2) one might deduct from the nitrogen in the urine of the period the nitrogen content of the Liebig extract given (0 723 − 0 156 = 0 567) which gives an hourly excretion of 0 136 gram, a more probable value A similar calculation for the urine of March 22 shows an hourly value of 0 142 gram of nitrogen

The metabolism of February 6 has been calculated on the basis of both methods of estimating the protein nitrogen elimination with the following results

Influence of small variations in protein metabolism upon the calculation of results

TIME	N IN URINE	NON-PROTEIN R Q	CALORIES	N IN URINE	NON-PROTEIN R Q	CALORIES
1 00–2 00	0 094	0 88	15 15	0 136	0 88	15 06
2 00–3 00	0 094	0 82	16 48	0 136	0 81	16 37
3 00–4 00	0 094	0 73	17 71	0 136	0 72	17 62
			49 34			49 05

This table shows that however crude the method of analysis may appear to the critical, the general result is practically uninfluenced by a possible error of 0 042 gram of estimated protein nitrogen elimination per hour

of 0 132 per hour, then 0 549 gram would be eliminated during four hours and ten minutes

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Influence of small variations in protein metabolism upon the calculation of results

TIME	N IN URINE	NON-PROTEIN R Q	CALORIES	N IN URINE	NON-PROTEIN R Q	CALORIES
1 00–2 00	0 094	0 88	15 15	0 136	0 88	15 06
2 00–3 00	0 094	0 82	16 48	0 136	0 81	16 37
3 00–4 00	0 094	0 73	17 71	0 136	0 72	17 62
			49 34			49 05

This table shows that however crude the method of analysis may appear to the critical, the general result is practically uninfluenced by a possible error of 0 042 gram of estimated protein nitrogen elimination per hour

B 25 grams of glycocoll

Since both carbon atoms contained in glycocoll are convertible into dextrose, it may be calculated that 25 grams of glycocoll produce 20 grams of dextrose In paper three it was shown that 20 grams of dextrose containing 75 calories caused, during the second hour only, an increase of 2 5 calories in heat production—a specific dynamic action of 3 3 The question was, would glycocoll behave in the same fashion?

The experiments were continued from the beginning of the second to the end of the ninth hour after the ingestion of the material, in two different series of four hours each and each series was done twice, making experiments which are essentially well agreeing duplicates (see table I) The important results appear in the following

Experiments 44 and 48

25 grams of glycocoll (4 66 grams N and 46 15 calories) in 150 cc of water plus 2 grams of Liebig's extract, at noon

Values in calories

TIME	PROTEIN	GLYCOCOLL	NON-PROTEIN	TOTAL CALCULATED	TOTAL FOUND
1 00–2 00	3 02	0 73	17 37	21 12	21 96
2 00–3 00	3 02	0 99	18 85	22 86	21 31
3 00–4 00	3 02	1 43	20 30	24 75	21 23
4 00–5 00	3 02	2 93	16 75	22 70	21 16
5 00–6 00	3 02	3 60	11 81	18 43	19 24
6 00–7 00	3 02	3 60	13 71	20 33	20 17
7 00–8 00	3 02	3 60	12 19	18 81	18 33
8 00–9 00	3 02	3 60	10 48	17 10	17 53
Total	24 16	20 48	121 46	166 10	160 93

The difference between calories calculated and calories found is 5 2 of which 3 6 falls in the single hour between three and four o'clock The result of the glycocoll ingestion was a decided rise in metabolism, which continued over a period of eight hours During the ninth hour the metabolism fell nearly to the basal value, which has been determined as 16 2 calories per hour For the eight hours at this level the basal metabolism would have been 129 6 calories Taking the lower figure, "calories found" in the

above experiment, the heat production after giving 25 grams of glycocoll amounted to 160 9 calories for a period of eight hours, an increase of 31 3 calories above the basal value Since the ingested glycocoll yields 46 15 calories in metabolism, it may be calculated that *the specific dynamic action of glycocoll is 70* Using a calculation (see below) which indicates that 8 per cent of the absorbed glycocoll is eliminated unchanged in the urine, the specific dynamic action may be estimated to be 74 This is calculated after the manner of Rubner If, however, one considers the very low level of glycocoll metabolism during the second and third hours as measured by the extra urea plus ammonia excretion, it is apparent that, although no considerable quantity of glycocoll is destroyed, yet the heat production is about at its maximum during these hours There is a rise of 5 calories above the basal metabolism, during the second hour, although the calories from glycocoll metabolism are indicated as being only 0 73 (or only 3 15 calories if the estimated glycocoll metabolism of the next two hours be included) In the interpretation of these results, valued information is to be obtained from the work of Folin and Denis [8] These authors find that one hour after the administration of about 2 grams of glycocoll to anesthetized cats, the "non-protein nitrogen" (which must contain absorbed glycocoll) has increased in the blood and in the tissues, but there has been no increase in the quantity of urea nitrogen in either locality The urea formation begins about an hour after the ingestion of the material From one experiment (in which the kidney function was undisturbed) the following details are reproduced

Glycocoll given, 1 867 grams, absorbed, 0 917 grams
Values, milligrams in 100 grams substance

TIME	CAROTID BLOOD		MUSCLE	
	UREA N	NON-PROTEIN N	UREA N	NON-PROTEIN N
At start	34	60	42	248
After 2 hours	44	100		
After 3 hours	50	101		
After 4 hours	60*	124*	54	304

Iliac artery

[8] Folin and Denis This *Journal*, xii, p 141, 1912, and their previous papers

It appears from this that the absorbed amino-acids circulate in the blood, and are retained in the muscle tissue, while after one hour the urea rises in the blood in response to the increased production of urea in the tissues These results are interpretative of the nitrogen figures representing the glycocoll metabolism in the dog as shown above, and indicate that they give an approximate idea of the actual happenings

Since there is a great rise in metabolism during the second hour after glycocoll ingestion and this is not accompanied by a large metabolism of absorbed glycocoll, it is evident that *glycocoll acts as a stimulus to increase the oxidative processes in the organism* Furthermore, since the high metabolism is found during the second hour when the urea production from glycocoll is still low, it appears that *the increased metabolism is due to the direct stimulus of glycocoll itself and not to any process concerned with intermediary metabolism such as the process of deamination*

Comparison to show the similarity between the metabolism after the ingestion of 20 grams of dextrose which exerts a specific dynamic action of 3 2 with that produced by its isoglucosic equivalent, 25 grams of glycocoll, which exerts a specific dynamic influence of 70, breaks down completely Most dogs drink a solution of dextrose of their own free will They drink a solution of glycocoll the first time it is offered to them, but not readily after that Glycocoll solutions are often vomited and some dogs never retain them Both materials have a sweet taste, and yet their action on the stomach wall, as well as upon the cells of the organism in general, is distinctly different It may be suggested that the action of glycocoll is merely through the sensory stimulation of the mucous membrane of the stomach and intestines, but the continuation of the increased metabolism for seven or eight hours after the ingestion of the material and long after the sensation of nausea has passed away, disproves this Furthermore, urea solutions which produce nausea and often vomiting, have no influence whatever upon metabolism as shown in paper three In the experiments here described, the dog betrayed no signs of nausea but in the two instances remained in the calorimeter without moving during the first hour of the experiment

In the work of Ringer and Lusk[9] it was shown that the nitrogen and sugar which arise from the ingestion of 20 grams of glycocoll by a phlorhizinized dog are usually completely eliminated in twelve hours In the present experiment it is found that the maximal action on metabolism takes place during the second to fifth hours, during which the mass action of the entering amino-acids may be greatest With the completion of the absorption and the destruction of the amino-acids as such, this mass action upon the cells diminishes with an accompanying diminution in the intensity of metabolism

It will be noted that the behavior of glycocoll is entirely similar to the behavior of meat, as has been set forth in paper two of this series Glycocoll, the simplest of the amino-acids, has the most profound effect of all those which have been studied How important the effect of glycocoll upon the general metabolism is, cannot, with certainty, be stated until it is known whether the large amount excreted after the administration of benzoate of soda[10] corresponds to the amount produced normally

The urinary analyses It has been admitted that the calculations of the hourly nitrogen values after administration of Liebig's extract could not be exactly calculated and the calculations become even more complex when amino-acids are given with Liebig's extract The following general procedure has been used (1) The values of total N and urea plus ammonia N were determined in hourly periods after giving Liebig's extract (2) Total N and urea plus ammonia N were determined in hourly periods for six hours after giving 25 grams of glycocoll plus Liebig's extract (3) These last values have been assumed as correct during the period when the dog was in the calorimeter (4) The protein metabolism of the time has been taken as an assumed value of the probable nitrogen elimination had no Liebig's extract or amino-acid been ingested (5) The difference between the urea plus ammonia nitrogen when Liebig's extract was given alone and when it was given with glycocoll, forms the basis of the determination of the amount of glycocoll metabolized (6) The increase in total nitrogen due to the addition of glycocoll to Liebig's extract, minus

[9] Ringer and Lusk *loc cit*

[10] Magnus-Levy *Münch med Wochenschr*, lii, p 2168, 1905, Ringer this *Journal*, x, p 327, 1911

the nitrogen of metabolized glycocoll, represents the amount of glycocoll which was eliminated unchanged

The above presents in general outline the method of all the determinations made with various amino-acids in this paper Exceptions to this procedure will be mentioned when they occur

The following table gives the results obtained after ingesting glycocoll

Urinary analyses after glycocoll ingestion

TIME	MARCH 28		MARCH 25		DIFFERENCE BETWEEN UREA PLUS NH_3 N = GLYCOCOLL N METABOLIZED	DIFFERENCE BETWEEN TOTAL N MINUS GLYCOCOLL N METABOLIZED = GLYCOCOLL N UNMETABOLIZED
	2 GRAMS LIEBIG S EXTRACT IN 150 CC WATER		25 GRAMS GLYCOCOLL PLUS 2 GRAMS LIEBIG S EXTRACT IN 150 CC WATER			
	Total N	Urea + NH_3 N	Total N	Urea + NH_3 N		
12 00–1 00	0 154	0 152	0 242	0 198	0 056	0 034
1 00–2 00	0 169	0 162	0 361	0 236	0 074	0 118
2 00–3 00	0 176	0 156	0 315	0 256	0 100	0 039
3 00–4 00	0 150	0 126	0 381	0 276	0 150	0 081
4 00–5 00	0 140	0 122	0 463	0 418	0 296	0 027
5 00–6 00	0 114	0 104	0 416	(0 348)		
Total	0 903	0 822 = 91 %	2 178	1 732 = 78 %	0 676	0 299

On the calorimeter days, March 29 (Exp 41) and April 4 (Exp 44), the actual elimination of nitrogen and the elimination assumed on the basis of the analyses of March 25 are given below

DATE	TIME	TOTAL N	UREA + NH_3 N	PER CENT
March 29, Exp 41	12 00–5 10	1 583	1 355	85
March 25 (est)	12 00–5 10	1 839	1 454	
April 4, Exp 44	12 00–5 08	1 825	1 578	86
March 25 (est)	12 00–5 08	1 823	1 403	

The results of the actual and calculated values of April 4 show little differences Greater differences are shown on March 29, the nitrogen elimination being 14 per cent less than on March 25 The assumed values were, however, adopted This was justified because the heat production in both experiments, 41 and 44, was essentially the same More perfect results than these could have been

obtained if the dog had been provided with a catheter and an apparatus for the automatic washing of the bladder at the end of each hourly period while in the calorimeter Although this could have been arranged, yet it was deemed undesirable on account of the necessity of keeping the dog in complete repose

On March 28, the day on which Liebig's extract alone was given, the total nitrogen was 0 114 and the urea plus ammonia N was 0 104 between five and six o'clock The value 0 114 N has been assumed as representing the fasting protein metabolism in the following experiments after glycocoll ingestion at noon

On April 13 and 23, the dog was placed in the calorimeter and his metabolism determined between five and nine o'clock The urine of the whole period was analyzed and was partitioned equally through the several hours The results were

DATE	EXP NO	TIME	TOTAL N	UREA + NH_3 N	PER CENT
April 13	48	4 00–9 10	2 425	2 297	95
		Per hour	0 469	0 445	
April 23	52	4 00–9 10	2 642	2 478	94
		Per hour	0 511	0 479	

Since the urea plus ammonia elimination when no glycocoll is ingested is 0 104 gram per hour (see above) the nitrogen elimination representing glycocoll metabolized would be in Experiment 48, 0 341 gram per hour, and in Experiment 52, 0 375 gram per hour The high percentage of urea plus ammonia nitrogen during this period of four hours shows that practically no glycocoll was eliminated in the urine as such, for, calculated on the basis before described, only 0 014 and 0 022 gram of glycocoll nitrogen per hour could be estimated in the urine The values of 0 469 and 0 511 gram of total N metabolized per hour between four and nine o'clock agree with the values found on March 28 of 0 463 and 0 461 for the two hours between four and six It is assumed that the urea elimination during the following three hours did not essentially vary from this

The method of determining the nitrogen elimination of the dog within the calorimeter as described above was also used in the following experiments with other amino-acids, and need not again be described

C 20 grams of i-alanine

Since 20 grams of *i*-alanine are convertible in the organism into 20 grams of dextrose, that quantity was given in Liebig's extract of beef and the metabolism determined in hourly periods from the second to fifth hour to discover whether its behavior was different from that of dextrose

The results were as follows

Experiment 43

20 grams of alanine (3 14 grams N and 60 7 calories) in 150 cc of water plus 2 grams of Liebig's extract at noon

Values in calories

TIME	PROTEIN	ALANINE	NON-PROTEIN	TOTAL CAL-CULATED	TOTAL FOUND
1 00–2 00	3 50	2 28	13 96	19 74	19 45
2 00–3 00	3 50	2 80	12 17	18 47	19 19
3 00–4 00	3 50	5 15	11 29	19 94	19 39
4 00–5 00	3 50	5 23		(19 26)	19 26
Total	14 00	15 46		77 41	77 29

The basal metabolism of the dog was 16 2 calories per hour or 64 8 calories during four hours Hence the ingestion of 20 grams of alanine caused an increase of 12 61 calories during four hours, which shows a specific dynamic action of 21 during this short period Folin and Denis (*loc cit*) have shown that there is no increase in urea in the blood during the first hour after alanine ingestion, but that the quantity of alanine increases in the blood During the second hour urea formation commences

It will be noticed that, although the quantity of alanine metabolized increased hour by hour, the heat production reached its maximum during the second hour

The behavior of alanine appears to be similar to that of glycocoll, but alanine acts as a less powerful stimulus Glycocoll caused a rise of 5 calories during the first hour, whereas alanine caused one of 3 5 calories

The urinary analyses The urinary analyses were computed as in the subjoined table

Urinary analyses after alanine ingestion

TIME	MARCH 28: 2 GRAMS LIEBIG S EXTRACT IN 150 CC WATER — Total N	MARCH 28: 2 GRAMS LIEBIG S EXTRACT IN 150 CC WATER — Urea + NH_3 N	APRIL 3: 20 GRAMS ALANINE PLUS 2 GRAMS LIEBIG S EXTRACT IN 150 CC WATER — Total N	APRIL 3: 20 GRAMS ALANINE PLUS 2 GRAMS LIEBIG S EXTRACT IN 150 CC WATER — Urea + NH_3 N	DIFFERENCE BETWEEN UREA PLUS NH_3 N = ALANINE N METABOLIZED	DIFFERENCE BETWEEN TOTAL N MINUS ALANINE N METABOLIZED = ALANINE NOT METABOLIZED
12 00–1 00	0 154	0 152	0 197	0 136	0	0 043
1 00–2 00	0 169	0 162	0 431	0 280	0 118	0 144
2 00–3 00	0 176	0 156	0 461	0 301	0 145	0 140
3 00–4 00	0 150	0 126	0 562	0 393	0 267	0 145
4 00–5 00	0 140	0 122	0 515	0 393	0 271	0 104
Total	0 789	0 718 = 91 %	2 166	1 503 = 70 %	0 801	0 576

The above figures show, in confirmation of Folin, that there is no urea production during the first hour after giving alanine There appears to be a large excretion of unoxidized alanine amounting in five hours to 18 per cent of the material ingested The metabolism of alanine as measured by the increase in urea plus ammonia N rose hour by hour

On the calorimeter day, April 1, Experiment 43, the hourly protein metabolism was assumed to be represented by 0 132 grams N, an amount which was eliminated on April 22, when no food was given at noon, the amino-acid metabolism was estimated from the computation shown above, which the following comparison justifies

DATE	EXPERIMENT	TIME	TOTAL N	UREA + NH_3 N	PER CENT
April 1	43	12 00–5 10	2 19	1 53	70
April 3	(est)	12 00–5 10	2 25	1 56	70

D 20 grams of i-glutamic acid

It has been stated that glutamic acid yields three of its carbon atoms as glucose in the diabetic organism, whereas two are oxidized Here appeared a chemical complex which might serve to

settle the theory of the specific dynamic action of protein The following serves to illustrate the results of its ingestion

Experiment 49

20 grams of glutamic acid (1 90 grams N and 62 9 calories) in 150 cc of water plus 2 grams of Liebig's extract at noon

Values in calories

TIME	PROTEIN	GLUTAMIC ACID	NON-PROTEIN	TOTAL CAL-CULATED	TOTAL FOUND
1 00–2 00	3 50	1 62	10 09	15 21	15 49
2 00–3 00	3 50	4 96	7 70	16 16	15 26
3 00–4 00	3 50	5 16	8 25	16 91	15 99
4 00–5 00	3 50	6 68	6 82	17 00	16 59
Total	14 00	18 42	32 86	65 28	63 33

The average between the calories "found" and "calculated" is 64 3 which may be compared with a basal metabolism of 64 8 calories for the period

Glutamic acid exerts no "specific dynamic action" whatsoever It is an amino-acid which is an important constituent of most protein and yet its ingestion does not increase the heat production, although it is freely absorbed and deaminized Therefore, *the process of deaminization and urea production are not concerned with the heat increase* accompanying specific dynamic action One may also draw the conclusion that *glutamic acid cannot yield glycocoll as an intermediary product*

If the behavior of glutamic acid is on account of its chemical configuration, then one must assume that *aspartic acid* behaves in a similar manner in metabolism, although no experiments have been made with this substance

The urinary analyses As in the last experiment the protein metabolized was assumed to be represented by a urinary elimination of 0 132 gram N per hour The remaining factors were computed as follows

Urinary analysis after glycocoll ingestion

TIME	MARCH 28 — 2 GRAMS LIEBIG'S EXTRACT IN 150 CC WATER		APRIL 16 — 20 GRAMS GLUTAMIC ACID PLUS 2 GRAMS LIEBIG'S EXTRACT IN 150 CC WATER		DIFFERENCE BETWEEN UREA PLUS NH_3 N = GLUTAMIC ACID METABOLIZED	DIFFERENCE BETWEEN TOTAL N MINUS GLUTAMIC ACID N = GLUTAMIC ACID UNMETABOLIZED
	Total N	Urea + NH_3 N	Total N	Urea + NH_3 N		
12 00–1 00	0 154	0 152	0 246	0 208	0 056	0 036
1 00–2 00	0 169	0 162	0 289	0 211	0 049	0 073
2 00–3 00	0 176	0 156	0 406	0 306	0 150	0 080
3 00–4 00	0 150	0 126	0 337	0 282	0 156	0 031
4 00–5 00	0 140	0 122	0 368	0 324	0 202	0 026
Total	0 789	0 718 = 91 %	1 646	1 331 = 81 %	0 613	0 246

Comparison of the analysis of the urine of April 17, the day on which the dog received glutamic acid and was placed in the calorimeter with that of April 16 with the same diet outside the calorimeter, showed the following relations

DATE	EXPERIMENT	TIME	TOTAL N	UREA + NH_3 N
April 17	49	12 00–5 10	1 509	1 397
April 16	(est)	12 00–5 10	1 707	1 387

E 20 grams of l-leucine

The following results were obtained after giving leucine

Experiment 50

20 grams of leucine (2 14 grams N and 118 9 calories) in 150 cc of water plus 2 grams of Liebig's extract at noon

Values in calories

TIME	PROTEIN	LEUCINE	NON PROTEIN	TOTAL CALCULATED	TOTAL FOUND
1 00–2 00	3 50	7 64	6 45	17 59	17 06
2 00–3 00	3 50	6 35	6 88	16 73	17 40
3 00–4 00	3 50	6 74	7 46	17 70	17 68
4 00–5 00	3 50	7 57	8 27	19 34	18 36
Total	14 00	28 30	29 06	71 36	70 50

It appears from the above, that in spite of its insolubility, a noticeable quantity of leucine was absorbed and metabolized during the first five hours after its ingestion Its action in raising the heat production was not great, the basal metabolism of 64 8 calories being increased by only 6 1 calories or about 10 per cent

The urinary analyses The value 0 132 gram N was again assumed to represent the nitrogen of the true protein metabolism of the time The other figures were determined as follows

Urinary analyses after leucine ingestion

TIME	MARCH 28 — 2 GRAMS LIEBIG S EXTRACT IN 150 CC WATER		APRIL 18 — 20 GRAMS LEUCINE PLUS 2 GRAMS LIEBIG S EXTRACT IN 150 CC WATER		DIFFERENCE BETWEEN UREA PLUS NH_3 N = LEUCINE METABOLIZED	DIFFERENCE BETWEEN TOTAL N MINUS LEUCINE N = LEUCINE NOT METABOLIZED
	Total N	Urea + NH_3 N	Total N	Urea + NH_3 N		
12 00–1 00	0 154	0 152	0 149	0 133	0	0
1 00–2 00	0 169	0 162	0 329	0 299	0 137	0 225
2 00–3 00	0 176	0 156	0 298	0 270	0 114	0 008
3 00–4 00	0 150	0 126	0 270	0 247	0 121	0
4 00–5 00	0 140	0 122	0 278	0 258	0 136	0 002
Total	0 789	0 718 = 91 %	1 324	1 207 = 91 %	0 508	0 035

The above shows no elimination of leucine nitrogen during the first hour but an elimination of a quarter of the nitrogen ingested during the following four hours Very little or no leucine could have been excreted as such in the urine

On April 19, the dog was given 20 grams of leucine with Liebig's extract and was then placed in the calorimeter The urine of this day in comparison with that of April 18 showed the following

DATE	EXPERIMENT	TIME	TOTAL N	UREA + NH_3 N	PER CENT
April 19	50	12 00–5 04	1 481	1 244	84
April 18	(est)	12 00–5 04	1 324	1 207	91

F 20 grams of tyrosine

The results of giving 20 grams of tyrosine are presented in the following

Experiment 46

20 grams of tyrosine (1 55 grams N and 109 9 calories) in 150 cc of water plus 2 grams of Liebig's extract at noon

Values in calories

TIME	PROTEIN	TYROSINE	NON-PROTEIN	TOTAL CALCULATED	TOTAL FOUND
1 00–2 00	3 50		14 43	17 93	16 56
2 00–3 00	3 50		14 12	17 62	17 20
3 00–4 00	3 50		16 39	19 89	18 56
4 00–5 00	3 50		14 68	18 18	17 14
Total	14 00		59 62	73 62	69 46

It appears from this table that the metabolism was slightly increased after giving 20 grams of tyrosine If the calories "found" and "calculated" be averaged, the result is 71 5 as against a basal value of 64 8 calories, a gain of 7 7 calories, or 10 per cent for the period The result is, therefore, similar to that obtained with leucine, except that in the case of leucine, there was a largely increased elimination of urea plus ammonia nitrogen which is not evident with tyrosine It is possible that tyrosine, even though only small quantities are absorbed, will act as a stimulus upon the cells (see Summary)

The urinary analyses After giving tyrosine, there was no increase in the nitrogen elimination during five hours which could not be ascribed to experimental error The following compares the results found in the experiment after giving 20 grams of tyrosine in Liebig's extract with the figures for Liebig's extract alone

DATE	EXPERIMENT	TIME	TOTAL N	UREA+NH_3 N	PER CENT
April 10	46	12 00–5 08	0 888	0 786	89
March 28	(est)	12 00–5 08	0 808	0 733	91

alanine, tyrosine and leucine in equal amounts and containing together 2 94 grams of N and 90 calories, exerts a more profound influence upon metabolism than does 100 grams of meat containing 3 grams of N and 78 protein calories, during an experimental period which includes the second to fourth or fifth hours after their ingestion The higher metabolism after giving the mixture of amino-acids may, in part, be due to the greater rapidity of their absorption and in part to the particular constituents of the mixture

The urinary analysis On April 9, 100 grams of beef-heart were given at noon and the hourly nitrogen excretion showed the following results

TIME	TOTAL N *grams*
12 00–1 00	0 208
1 00–2 00	0 343
2 00–3 00	0 397
3 00–4 00	0 334
4 00–5 00	0 407
Total	1 689

On April 11, when the dog was in the calorimeter, 1 466 grams of N were eliminated between the hours of twelve and five o'clock

IV GENERAL STATISTICS

In the above research, various amino-acids have been given under such conditions that their action during the second to fifth hours after ingestion are strictly comparable The following table gives a summary of the results

urine during the first hour after the ingestion of amino-acids, the urea plus ammonia nitrogen was divided by 4 2 hours to determine the average hourly metabolism of amino-acid, which gave the value of 0 258 gram of N

II 100 grams of meat

In paper two it was shown that ingestion of 1000 grams of meat by the same dog used in these experiments, caused the metabolism to rise from 16 2 to 30 calories per hour The influence of 100 grams of meat from beef heart is indicated below

Experiment 47

100 grams of meat (3 0 grams N and 78 protein calories)

Values in calories

TIME	PROTEIN	NON PROTEIN	TOTAL CALCULATED	TOTAL FOUND
1 00-2 00	9 09	10 37	19 46	20 02
2 00-3 00	10 52	9 68	20 20	19 81
3 00-4 00	8 85	11 91	20 70	18 80
Total	28 46	31 99	60 45	58 63

The average metabolism during three hours may be estimated at 59 55 calories, an increase of 11 above the basal metabolism Assuming for the sake of comparison, that the metabolism during a fourth hour is maintained at the average height of 19 8 calories hourly, as one may on account of the nitrogen in the urine during this hour, the total metabolism may be estimated at 79 3 calories during four hours, and the results may be compared with those obtained after giving 27 5 grams of mixed amino-acids, as follows

	100 grams meat (3 grams N and 78 protein calorie)	27 5 grams mixed amino-acids (3 46 grams N and 107 calories)
Calories in four hours	79 3	84 4
Increase above basal value	14 5	19 6

Since, as has been said, glutamic acid exerts no heat increasing function, it may be concluded that *22 grams made up of glycocoll,*

Table indicating the specific dynamic action of various amino-acids, separately, together and compared with meat, during an interval of four hours

SUBSTANCE	CALORIES OF INGESTA	CALORIES OF METABOLISM	INCREASE IN CALORIES OF METABOLISM	100 CALORIES OF INGESTA CAUSES INCREASE IN METABOLISM IN CALORIES
No food	0	64 8		
25 grams glycocoll (4 66 grams N)	46 15	85 7	20 9	45
20 grams alanine (3 14 grams N)	60 7	77 4	12 6	21
20 grams glutamic acid (1 90 grams N)	62 9	64 3	0 0	0
20 grams leucine (2 14 grams N)	118 9	70 9	6 1	5
20 grams tyrosine (1 55 grams N)	109 9	71 5	7 7	7
5 5 grams of each of above (3 46 grams N)	107 1	84 4	19 6	19
100 grams meat (3 grams N)	78 0	79 3*	14 5	19

* Last hour estimated

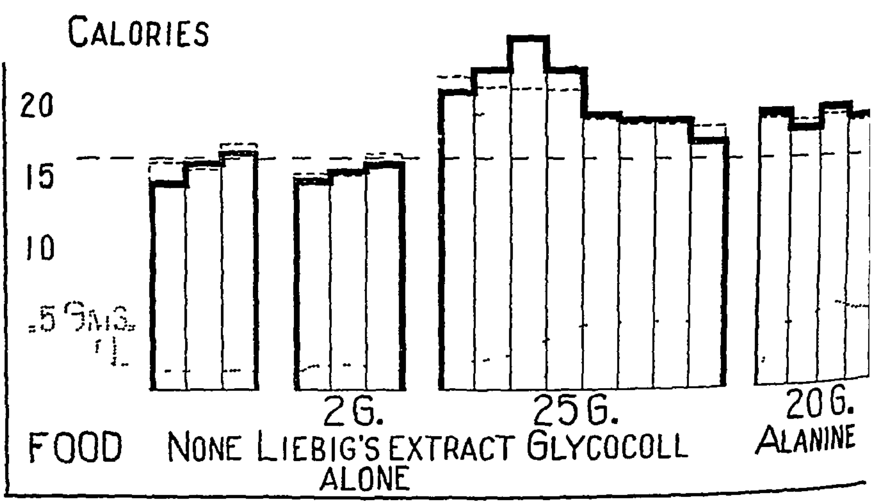
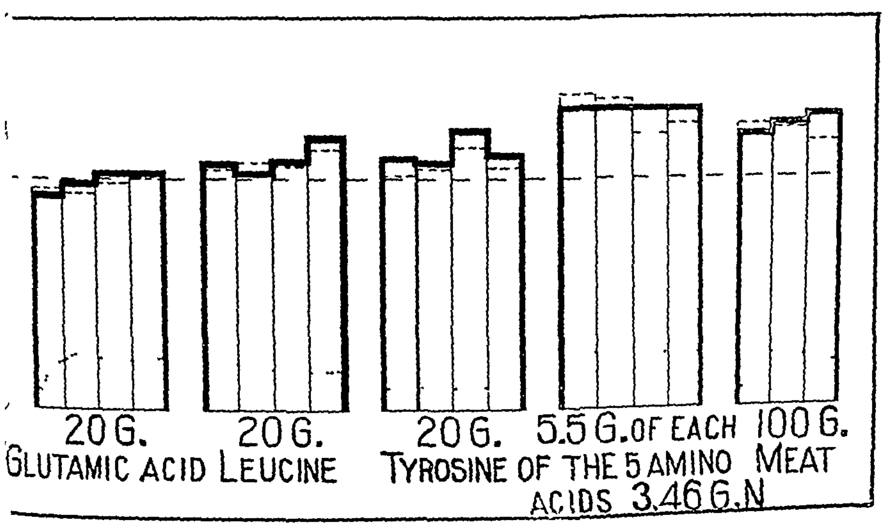


CHART 1 —DOG II HOURLY METABOLISM BEGINNING THE SECO
Heavy line—Calories calculated Broken line—Calories found Dotted line—Nitrog

The preceding table shows that the simplest amino-acid, glycocoll, has the most powerful action and that alanine also has a pronounced effect Leucine and tyrosine, per gram of substance, exert about half the effect of alanine, while glutamic acid, with its two carboxyl groups, is without influence The increase in the number of calories after giving 27 5 grams of the mixed acids is almost as great as when 25 grams of glycocoll were given The accompanying chart shows many of the essential features of the work

The metabolism of the amino-acids was at a higher level when they were mixed together than when they were given alone, as is indicated by the following table

	Urinary N from amino-acids metabolized during five hours *grams*
Glycocoll	0 676
Alanine	0 801
Glutamic acid	0 613
Leucine	0 508
Tyrosine	0 0
Mixed amino-acids	1 084



ND HOUR AFTER GIVING AMINO-ACIDS WITH LIEBIG'S EXTRACT
en of protein plus amino-acid metabolized

tration of tyrosine, yet by a colorimetric test they were able to unmistakably prove the presence of tyrosine, in both blood and muscle

3 A mixture, made up of 5 5 grams of each of the five amino-acids, glycocoll, alanine, glutamic acid, leucine and tyrosine, showed a more rapid metabolism of the constituent amino-acids than when they were given alone, and the effect on metabolism was as pronounced as that seen after giving 25 grams of glycocoll It was greater than that of 100 grams of meat which contained about the same quantity of nitrogen

4 The influence of glycocoll and alanine are not due to the fact that they produce nausea, for urea solutions also produce nausea but have no effect on the metabolism

The influence is not due to movements of the intestines, for Benedict and Emmes[13] have shown that diarrhœa produced by saline cathartics does not influence metabolism Nor is the influence of glycocoll due to excitation of the digestive glands, for Liebig's extract of beef, which is known to induce glandular activity, is without influence upon the general heat production

5 Since glycocoll and alanine are completely convertible into dextrose in phlorhizin glycosuria, and since the production of sugar from protein has been shown to be a normal function (see paper two), it follows, from Rubner's hypothesis, that glycocoll and alanine should exert no more influence upon metabolism than would a corresponding quantity of glucose However, it has been shown that these two amino-acids, which are completely convertible into sugar, cause a very great increase in heat production They do not act like sugar, into which they are convertible, but they act in virtue of their own special configuration They do not act through the metabolism products which they form, for these are practically without effect as heat-increasing factors, but they act as stimuli upon the protoplasm with which they come in contact

6 The work of Howland[14] accomplished in this laboratory, has shown that an infant seven months old when given a modified milk administered in six equal portions during twenty-four hours and containing 3 58 grams of nitrogen in the day's diet produced during sleep 14 91 calories per hour When 30 grams of casein

[13] Benedict and Emmes *Amer Journ of Physiol*, xxx, p 197, 1912

[14] Howland *Zeitschr f physiol Chem*, lxxiv, p 1, 1911

were added to this diet and the nitrogen of the ingesta was 7 85 grams, the metabolism of the sleeping infant rose to 18 81 calories per hour, an increase of 3 9 calories or 26 per cent more than its former value The casein added to the diet was the equivalent of 5 5 calories per hour The urine of the period before the high protein diet contained 2 20 grams of N per day, and, during the day of the high protein diet, 4 46, an increase of 2 26 grams This increase corresponds to 2 6 calories per hour An increase in protein metabolism of 2 6 calories per hour brought about an increase of 3 9 calories in the metabolism of the sleeping infant This is the only instance in which the metabolism may be seen to be permanently raised above the calorific value of the extra protein metabolism The explanation may lie in the fact that the youthful protoplasm is more sensitive to stimuli than that of the adult In Dog I, the basal metabolism was 759 calories per square meter of surface in twenty-four hours, in Dog II it was 784, in a dwarf nineteen years old it was 840, whereas in sleeping infants of three and seven months, it ranged between 1000 and 1100 These results could be obtained on account of comparable conditions of perfect rest

As a final conclusion, it may be stated that *the increase in metabolism after the ingestion of meat is due to the mass action of amino-acids acting as stimuli upon the cellular protoplasm*

T TEMPERATURE		MORNING WEIGHT	BEHAVIOR OF DOG	REMARKS
End	Difference			
		kg		
38 50	+0 02	9 0	Very quiet	2 grams Liebig's extract of beef in 150 cc water at noon
38 50	0 0		Quiet	
38 53	0 03		Moving 2 min	
37 85	0 0	9 5	Very quiet	2 grams Liebig's extract in 150 cc water at noon
37 74	−0 11		Quiet	
37 82	+0 08		Quiet	
38 52	+0 30	9 7	Very quiet	25 grams glycocoll (4 66 grams N) in Liebig's extract at noon
38 63	+0 11		Moving 1 min	
38 32	−0 30		Restless 3 min	
38 42	+0 10		Moving 5 min	
38 39	+0 42	9 7	Very quiet	25 grams glycocoll in Liebig's extract at noon
38 34	−0 05		Quiet	
38 25	−0 09		Quiet	
38 41	+0 06		Moving 2 min	
38 27	+0 05	9 5	Quiet	25 grams glycocoll in Liebig's extract at noon
38 29	+0 02		Moving 1 min	
38 29	0 0		Very quiet	
38 20	−0 09		Quiet	
37 84	+0 01	9 4	Quiet	25 grams glycocoll in Liebig's extract at noon
37 81	−0 03		Quiet	
37 83	+0 02		Moving 4 min	

TABLE 1 (Continued)

O- N IE	NON PROTEIN			CALORIES					BO	
	CO_2	O_2	R Q	Protein	Amino-acid	Non-Protein	Total Calculated	Total Found	Start	
h	gram	gram								
8	5 18	4 01	0 94	3 50	2 28	13 96	19 74	19 45	38 25	g
15	4 60	3 48	0 96	3 50	2 80	12 17	18 47	19 19		0
7	4 12	3 26	0 92	3 50	5 15	11 29	19 94	19 39		0
1				3 50	5 23		(19 26)	19 26		0
1				14 00	15 46		77 41	77 29		0
										0
9	3 92	2 72	1 05	3 50	1 62	10 09	15 21	15 49	37 69	
0	2 77	2 23	0 91	3 50	4 96	7 70	16 16	15 26		0
6	2 48	2 52	0 72	3 50	5 16	8 25	16 91	15 99		0
2	2 01	2 08	0 70	3 50	6 68	6 82	17 00	16 59		0
7				14 00	18 42	32 86	65 28	63 33		0
										0
7	2 24	1 89	0 86	3 50	7 64	6 45	17 59	17 06	37 60	
4	2 33	2 03	0 83	3 50	6 35	6 88	16 73	17 40		0
1	2 32	2 25	0 75	3 50	6 74	7 46	17 70	17 68		0
6	2 45	2 52	0 71	3 50	7 57	8 27	19 34	18 36		0
8				14 00	28 30	29 06	71 36	70 50		0
										0
	4 97	4 24	0 85	3 50		14 43	17 93	16 56	37 70	
	4 81	4 16	0 84	3 50		14 12	17 62	17 20		
	5 28	4 89	0 79	3 50		16 39	19 89	18 56		
	4 78	4 38	0 79	3 50		14 68	18 18	17 14		
				14 00		59 62	73 62	69 46		
8	3 41	2 40		3 50	8 06	9 81	21 37	22 27	37 85	
8	3 61	3 69		3 50	8 06	9 81	21 37	21 77		0
8	2 88	2 09		3 50	8 06	9 81	21 37	19 25		0
25	2 91	3 50		3 50	8 06	9 81	21 37	20 09		0
32	12 81	11 68	0 80	14 00	32 24	39 24	†85 48	83 38		0
										1
	3 84	2 98	0 94	9 09		10 37	19 46	20 02	38 06	
	3 29	2 85	0 84	10 52		9 68	20 20	19 81		
	3 50	3 64	0 70	8 85		11 94	20 79	18 80		
				28 46		31 99	60 45	58 63		

f Amino-acids upon Metabolism

...DY TEMPERATURE		MORNING WEIGHT	BEHAVIOR OF DOG	REMARKS
End	Difference			
		kg		
38 41	+0 16	9 7	Quiet	20 grams alanine (3 14 grams N) in Liebig's extract at noon
38 52	+0 11		Quiet	
38 46	−0 06		Quiet	
38 46	0 0		Restless 15 m	
37 60	−0 09	9 7	Quiet	20 grams glutamic acid (1 90 grams N) in Liebig's extract at noon
37 49	−0 11		Quiet	
37 60	+0 11		Quiet	
37 71	+0 11		Quiet	
37 67	+0 07	9 5		20 grams leucine (2 14 grams N) in Liebig's extract at noon
37 72	+0 05			
37 79	+0 05			
37 81	+0 02			
37 67	−0 03	9 7	Quiet	20 grams tyrosine (1 55 grams N) in Liebig's extract at noon
37 76	+0 09		Quiet	
37 74	−0 02		Moving 7 min	
37 78	+0 04		Restless	
38 22	+0 37	9 7	Very quiet	5 5 grams each of glycocoll, alanine glutamic acid, leucine and tyrosine in Liebig's extract at noon
38 20	−0 02		Quiet	
38 37	+0 17		Quiet	
38 40	+0 03		Quiet	
38 22	+0 16	9 6	Quiet	100 grams beef-heart at noon
37 92	−0 30		Quiet	
38 06	+0 14		Moving 1 min	

DATE 1912		EXP NO	TIME	CO_2	O_2	R Q	H_2O	PROTEIN N IN URINE	A… N U
			p m	gram	gram		gram	gram	g
pril	1	43	1 00–2 00	7 34	5 93	0 90	8 85	0 132	0
			2 00–3 00	6 97	5 59	0 91	8 32	0 132	0
			3 00–4 00	7 45	6 21	0 87	8 32	0 132	0
			4 00–5 00	6 99			8 23	0 132	0
			Total	28 75				0 528	0
pril	17	49	1 00–2 00	5 86	4 34	0 98	12 04	0 132	0
			2 00–3 00	6 13	4 98	0 90	10 65	0 132	0
			3 00–4 00	5 93	5 25	0 82	9 10	0 132	0
			4 00–5 00	6 11	5 28	0 84	8 47	0 132	0
			Total	24 03	19 85			0 528	0
pril	19	50	1 00–2 00	5 84	5 35	0 79	9 88	0 132	0
			2 00–3 00	5 53	5 10	0 79	9 11	0 132	0
			3 00–4 00	5 64	5 44	0 75	8 30	0 132	0
			4 00–5 00	6 03	5 97	0 73	8 28	0 132	0
			Total	23 04	21 86			0 528	0
April	10	46	1 00–2 00	6 20	5 35	0 84	8 56	0 132	
			2 00–3 00	6 04	5 27	0 83	7 66	0 132	
			3 00–4 00	6 51	6 00	0 79	7 60	0 132	
			4 00–5 00	6 01	5 49	0 80	7 10	0 132	
			Total	24 76	22 11			0 528	
April	8	45	1 00–2 00	7 69	6 08	0 92	8 25	0 132	0
			2 00–3 00	7 89	7 37	0 78	8 74	0 132	0
			3 00–4 00	7 16	5 77	0 90	8 07	0 132	0
			4 00–5 00	7 19	7 18	0 73	8 28	0 132	0
			Total	29 93	26 40			0 528	1
April	11	47	1 00–2 00	7 05	5 88	0 87	9 71	0 343	
			2 00–3 00	7 00	6 20	0 82	8 96	0 397	
			3 00–4 00	6 62	6 46	0 75	8 02	0 334	
			Total						

† Calculated f… whole period

to 7 1 calories or 44 per cent From the respiratory quotient it is evident that the greater part of the oxidative processes was concerned with the metabolism of carbohydrate

The urinary analyses Following the ingestion of the standard diet at noon, the urine was analyzed in hourly periods with the following results

Elimination of total N in grams after standard diet at noon

TIME	NOV 23	NOV 27	NOV 29	JAN 8	DEC 12 EXP 5	DEC 13	JAN 9 EXP 10
12 00–1 00	0 146	0 154	0 209	0 154		0 187	
1 00–2 00	0 142	0 171	0 202	0 182		0 194	
2 00–3 00	0 239	0 225	0 247	0 215		0 230	
3 00–4 00	0 229	0 241	0 255	0 208		0 240	
4 00–5 00	0 251	0 361	0 282	0 236		0 250	
Total	1 007	1 155	1 195	0 995	1 081*	1 101	0 932†

* 12 to 5 15 p m † 12 to 5 09 p m

It is evident, from this, that the hourly nitrogen output shows a constantly rising tendency The values assumed for the calorimeter periods are approximate only, but it has already been set forth that a difference of 0 042 gram of nitrogen per hour makes almost no difference in the calculation of the heat production, and this latter is the essential point on the days when the calorimeter was used

When 100 grams of meat were given alone instead of with 100 grams of biscuit meal and 20 grams of fat, the excretion of nitrogen in the urine was much more rapid as appears below (see also fifth paper)

April 9 1912	Total N
12 00–1 00	0 208
1 00–2 00	0 343
2 00–3 00	0 397
3 00–4 00	0 334
4 00–5 00	0 407
Total	1 689

This can be due (1) to a more rapid absorption of protein or (2) to a more rapid destruction of amino-acids within the organism in the absence of carbohydrates

B The standard diet minus 67 grams of meat

Beginning December 2, only 33 grams instead of 100 grams of meat were for a time added to the standard diet On December 6 the metabolism was determined with the following results

Experiment 4

Standard diet minus 67 grams of meat at noon

TIME	N IN URINE	NON PROTEIN R Q	CALORIES	
			Calculated	Found
1 00–2 00	0 121	0 90	22 61	20 47
2 00–3 00	0 121	0 89	22 12	21 81
3 00–4 00	0 185	0 91	23 69	22 99
Total			68 42	65 27
Per hour			22 80	

Again, the greater part of the non-protein calories is obtained from carbohydrate The metabolism is increased from the basal value of 16 2 calories to 22 8, a rise of 6 6 calories or 41 per cent

The metabolism is only 2 per cent less than the average metabolism after giving the full standard diet The withdrawal of 67 grams of meat is, therefore, almost without influence on the metabolism, although it has been shown in paper five that 100 grams of meat ingested alone caused the hourly metabolism to rise from 16 2 to 20 2 calories, an increase of 4 calories or 25 per cent

The urinary analyses See next section

C The standard diet minus 67 grams of meat plus 20 grams of glutamic acid

In this experiment, 20 grams of glutamic acid were added to the diet last employed so that the 1 9 grams of N contained in the glutamic acid might replace the 2 grams of N contained in the 67 grams of meat withdrawn from the standard diet The results of the calorimeter experiments were as follows

Experiment 6

Standard diet minus 67 grams of meat plus 20 grams of glutamic acid at noon

TIME	N IN URINE	NON PROTEIN R Q	CALORIES	
			Calculated	Found
1 00–2 00	0 135	0 95	22 32	22 42
2 00–3 00	0 184	0 95	22 30	22 37
3 00–4 00	0 205	0 95	23 26	23 08
4 00–5 00	0 239	0 96	22 92	21 62
Total			90 80	89 49
Average			22 70	

The heat production is mostly at the expense of carbohydrate The addition of glutamic acid was without influence upon the metabolism, which was measured as 22 7 calories per hour in contrast with 22 8 calories obtained before This accords with the results shown in paper five, which demonstrated that glutamic acid exerted no influence upon heat production

The urinary analyses The analyses of the urines eliminated after giving the standard diet minus 67 grams of meat with and without 20 grams of glutamic acid are shown below

Total N in urine after standard diet minus 67 grams of meat

TIME	DEC 4	DEC 5 EXP 4	DEC 6	PLUS 20 GRAMS GLUTAMIC ACID	
				DEC 14	DEC 15 EXP 6
12 00–1 00	0 150		0 101	0 107	
1 00–2 00	0 135		0 122	0 185	
2 00–3 00	0 184		0 122	0 197	
3 00–4 00	0 205		0 185	0 250	
4 00–5 00	0 239		0 157	0 275	
Totals	0 913	0 642*	0 687	1 014	0 870†

* 12 to 5 06 † 12 to 5 10

The value obtained on December 15 was not sufficient to indicate with certainty that there was any extra excretion of nitrogen during five hours after the ingestion of the diet containing 20

grams of glutamic acid The urine of the period contained 0 807 gram, of which 93 per cent was urea plus ammonia nitrogen

From January 2 to 4, the standard diet was given at six p m , at noon on January 3, 50 grams of dextrose were ingested and two days later 50 grams of dextrose plus 20 grams of glutamic acid were administered The following table shows the quantity of total nitrogen in the urine

TIME	JANUARY 3	JANUARY 5
	50 GRAMS DEXTROSE	50 GRAMS DEXTROSE + 20 GRAMS GLUTAMIC ACID
12 00–1 00		0 067
1 00–2 00		0 111
2 00–3 00		0 198
3 00–4 00		0 188
4 00–5 00		0 149
Total	0 7273*	0 713

* 12 to 5 20

Since the nitrogen of glutamic acid readily appears in the urine of a phlorhizinized dog or in the urine of a dog which has been given it with Liebig's extract, it seems that, when given with sugar, it must either (1) spare protein from destruction or (2) form a synthetic compound with carbohydrate either with glucose or with glycogen within the organism This accords with the work of Luthje, Murlin and others (see second paper)

In the calculation of those experiments in which glutamic acid was given with a mixed diet, the N elimination was attributed to protein metabolism

Summarizing the total nitrogen excretion for five hours thus far discovered, the following results are found

DATE	TIME	DIET	TOTAL N
Jan 30, 1912	12 00–5 00	No food	0 650
Apr 9, 1912	12 00–5 00	No food	0 660
Nov 23, 1911	12 00–5 00	Standard diet	1 007
Nov 27, 1911	12 00–5 00	Standard diet	1 155
Nov 29, 1911	12 00–5 00	Standard diet	1 195
Nov 12, 1911	12 00–5 15	Standard diet	1 081
Nov 13, 1911	12 00–5 00	Standard diet	1 101
Jan 8, 1912	12 00–5 00	Standard diet	0 995
Jan 9, 1912	12 00–5 09	Standard diet	0 932
Dec 4, 1911	12 00–5 00	Standard diet—67 grams meat	0 913
Dec 5, 1911	12 00–5 06	Standard diet—67 grams meat	0 642
Dec 6, 1911	12 00–5 00	Standard diet—67 grams meat	0.687
Dec 14, 1911	12 00–5 00	Standard diet – 67 grams meat + 20 grams glutamic acid	1 014
Dec 15, 1911	12 00–5 10	Standard diet – 67 grams meat + 20 grams glutamic acid	0 870

These experiments, by themselves, would confirm Rubner's view that the specific dynamic action of protein does not appear when protein is added to a mixed diet, because the added protein merely tends to replace the ordinary wear and tear quota of protein metabolism and is not quickly destroyed as happens when it is ingested alone Hence, small variations in the amount of protein ingested would cause no change in the metabolism, as indeed these experiments tend to indicate

The following work, however, establishes another view-point

D One-half portion of the standard diet

It has been shown that the ingestion of the standard diet with its 100 grams of meat or the same with only 33 grams, or, 33 grams of meat plus 20 grams of glutamic acid, resulted in the same metabolism It was decided to reduce the quantity of food that the action of the amino-acid in metabolism might be more strongly revealed To this end, the standard diet was divided into two portions, Portion I and Portion II, each containing 50 grams of biscuit meal, 10 grams of lard and 5 grams of bone ash, in addition, Portion I contained 33 grams of meat while Portion II contained 67 grams of meat Portion I was used exclusively as the diet for the experimental period and was given at noon daily, while Portion II was given at 6 p m

E Portion I

The influence of a half-portion of the standard diet known as Portion I, is shown in the following experiments

Experiments 25 and 27

Portion I at noon

TIME	N IN URINE	NON PROTEIN R Q	CALORIES		N IN URINE	NON PROTEIN R Q	CALORIES	
			Cal culated	Found			Cal- culated	Found
1 00–2 00	0 202	0 90	22 59	22 75	0 136	0 91	20 71	21 13
2 00–3 00	0 260	0 84	24 39	23 46	0 161	0 92	20 77	21 44
3 00–4 00	0 278	0 80	20 54	20 20	0 213	0 80	21 95	19 69
4 00–5 00	0 248	0 91	18 24	18 70	0 188	0 96	17 86	20 04
5 00–6 00					0 188	0 86	21 88	20 25
Total			85 76	85 11			103 17	102 55
Per hour			21 44				20 63	

Since the dog moved during a period of eight minutes, between two and three o'clock in Experiment 25, thereby causing the metabolism to increase 2 calories during this hour, the value 20 6 calories per hour, as found in Experiment 27, may be accepted as the true level of metabolism during the second to sixth hour after the ingestion of Portion I The increase above the basal value of 16 2 calories was, therefore, 4 4 calories or 27 per cent per hour For the whole period, the increase amounted to 22 7 calories It may here be recalled that 50 grams of dextrose given to this same dog, increased the metabolism 20 per cent in the second hour after its ingestion, but it caused a total increase of only 8 8 calories Portion I, consisting of 50 grams of biscuit meal, 33 grams of meat and 10 grams of fat, has a more powerful and more prolonged influence over the production of heat To what can this be attributed? In part perhaps to the more prolonged period of absorption due to the time required for the digestion of starch This is illustrated by the fact that the respiratory quotient indicates that carbohydrate is not so much a dominating factor in the metabolism as when 50 grams of

DATE	TIME	DIET	TOTAL N
Jan 30, 1912	12 00-5 00	No food	0 650
Apr 9, 1912	12 00-5 00	No food	0 660
Nov 23, 1911	12 00-5 00	Standard diet	1 007
Nov 27, 1911	12 00-5 00	Standard diet	1 155
Nov 29, 1911	12 00-5 00	Standard diet	1 195
Nov 12, 1911	12 00-5 15	Standard diet	1 081
Nov 13, 1911	12 00-5 00	Standard diet	1 101
Jan 8, 1912	12 00-5 00	Standard diet	0 995
Jan 9, 1912	12 00-5 09	Standard diet	0 932
Dec 4, 1911	12 00-5 00	Standard diet—67 grams meat	0 913
Dec 5, 1911	12 00-5 06	Standard diet—67 grams meat	0 642
Dec 6, 1911	12 00-5 00	Standard diet—67 grams meat	0,687
Dec 14, 1911	12 00-5 00	Standard diet − 67 grams meat + 20 grams glutamic acid	1 014
Dec 15, 1911	12 00-5 10	Standard diet − 67 grams meat + 20 grams glutamic acid	0 870

These experiments, by themselves, would confirm Rubner's view that the specific dynamic action of protein does not appear when protein is added to a mixed diet, because the added protein merely tends to replace the ordinary wear and tear quota of protein metabolism and is not quickly destroyed as happens when it is ingested alone Hence, small variations in the amount of protein ingested would cause no change in the metabolism, as indeed these experiments tend to indicate

The following work, however, establishes another view-point

D One-half portion of the standard diet

It has been shown that the ingestion of the standard diet with its 100 grams of meat or the same with only 33 grams, or, 33 grams of meat plus 20 grams of glutamic acid, resulted in the same metabolism It was decided to reduce the quantity of food that the action of the amino-acid in metabolism might be more strongly revealed To this end, the standard diet was divided into two portions, Portion I and Portion II, each containing 50 grams of biscuit meal, 10 grams of lard and 5 grams of bone ash, in addition, Portion I contained 33 grams of meat while Portion II contained 67 grams of meat Portion I was used exclusively as the diet for the experimental period and was given at noon daily, while Portion II was given at 6 p m

Experiment 30

50 grams of biscuit meal at noon

TIME	N IN URINE	NON PROTEIN R Q	CALORIES	
			Calculated	Found
1 00-2 00	0 182	1 00	18 75	18 54
2 00-3 00	0 182	0 96	18 58	18 15
3 00-4 00	0 182	0 98	19 31	18 69
4 00-5 00	0 182	0 88	19 95	18 35
Total			76 59	73 73
Per hour			19 15	

It is evident from the above, that the 50 grams of biscuit meal induces a metabolism of about 19 15 calories per hour, in contrast with 16 2 calories without food and 20 6 calories with Portion I

The urinary analyses On February 23, 50 grams of biscuit meal were given at noon and the urine was collected in hourly periods and was analyzed with the following results

Time	Total N
12 00-1 00	0 104
1 00-2 00	0 149
2 00-3 00	0 160
3 00-4 00	0 146
4 00-5 00	0 154
Total	0 713

On the day of the experiment, however, the urine between noon and 5 05 p m contained 0 930 gram N or 0 182 per hour The comparative constancy of the results in hourly periods between one and five o'clock in the first experiment, led to the apportionment of the nitrogen of the calorimeter period equally among the several hours of the calorimeter experiment

G 50 grams of biscuit meal plus 10 grams of lard

The results obtained after giving 50 grams of biscuit meal plus 10 grams of lard are given below

Experiment 31

50 grams of biscuit meal plus 10 grams of lard

TIME	N IN URINE	NON PROTEIN R Q	CALORIES	
			Calculated	Found
1 00–2 00	0 140	0 90	17 37	17 99
2 00–3 00	0 166	0 93	19 79	18 62
3 00–4 00	0 166	0 90	20 09	19 47
Total			57 25	56 08
Per hour			19 08	

The result of this experiment shows that *the heat production is uninfluenced by the addition of 10 grams of lard to 50 grams of biscuit meal* The respiratory quotient is lowered by the ingestion of fat but Rubner's isodynamic law holds true, and the total heat production is not increased

The urinary analyses On February 28, the urine showed the following analytical results after giving 50 grams of biscuit meal plus 10 grams of lard

Time	Total N
12 00–1 00	0 1306
1 00–2 00	0 2078
2 00–3 00	0 2585
3 00–4 00	0 2503
Total	0 8470

Since the urine of February 29 during the period from noon till 4 07 p m contained only 0 677 gram of nitrogen, nitrogen values obtained after giving 50 grams of biscuit meal on February 27 were employed

H Analysis of the effect of Portion I

The following table summarized the results obtained after giving Portion I and fractions thereof

DIET	N IN URINE PER HOUR	CALCULATED PER HOUR
No food	0 132	16 2
50 grams biscuit meal	0 182	19 15
50 grams biscuit meal + 10 grams lard	0 164	19 08
50 grams biscuit meal + 10 grams lard + 33 grams meat	0 170	20 63

It is evident that the addition of a small quantity of fat has no influence on the heat production, whereas as small an amount of meat as 33 grams very considerably increases the metabolism The increase in metabolism caused by meat ingestion is, however, in no way accompanied by an increased protein metabolism as indicated by comparison of the average hourly output of urinary nitrogen during the calorimeter periods It has already been shown in the last paper, that 100 grams of meat (3 grams N) caused the production of 20 2 calories per hour in this dog, so that it may be that the amino-acids from 33 grams (1 gram N) of meat, together with those contained in 50 grams of biscuit meal (1 1 grams N), may be largely determinative of the height of metabolism (20 6 calories) when Portion I is given This would be true if the cells were stimulated to increased oxidation by amino-acids In presenting this idea, it is not forgotten that Rubner's interpretation, as regards the cause of the rise in heat production, also explains the phenomenon

The truth of the matter, however, was to be laid bare after the completion of experiments involving the ingestion of amino-acids with Portion I

I 20 grams of glutamic acid plus Portion I

As was to be expected from the former work, glutamic acid showed no power to increase metabolism when it was given with Portion I The results of an experiment illustrating this is given here

Experiment 26

20 grams of glutamic acid plus Portion I at noon

TIME	N IN URINE	NON PROTEIN R Q	CALORIES	
			Calculated	Found
1 00–2 00	0 202	1 03	20 80	21 66
2 00–3 00	0 260	0 96	19 08	19 76
3 00–4 00	0 278	0 72	18 69	18 11
4 00–5 00	0 248	0 90	21 96	19 76
Total			80 53	79 29
Per hour			20 13	

The value 20 13 calories per hour agrees well with 20 6 calories found with Portion I alone on February 19

The urinary analyses The same condition of affairs as was found to exist after giving glutamic acid with the standard diet, was revealed in the analysis of the urine after giving Portion I with glutamic acid, that is to say, there was no certain increase in the amount of nitrogen in the urine This is shown in the following

Urinary analyses after giving Portion I plus 20 grams of glutamic acid at noon

TIME	FEBRUARY 15		FEBRUARY 16	
	TOTAL N	UREA + NH_3 N	TOTAL N	UREA + NH_3 N
10 00–11 00	0 103		0 139	
Intermission				
12 00–1 00	0 123	0 107		
1 00–2 00	0 157	0 143		
2 00–3 00	0 208	0 184		
3 00–4 00	0 180	0 156		
4 00–5 00	0 282	0 262		
Total	0 950	0 852	1 234*	1 130
Per cent		89		91

* Urine from noon to 5 08 p m

Although the urine obtained on the calorimeter day, contained more nitrogen than on the day previous, it did not contain more than on February 12, when Portion I alone was given The hourly figures of February 12 were, therefore, adopted as representing the protein metabolism of February 16 On the latter date 7 15 grams of N were given including that contained in Portions I and II and 20 grams of glutamic acid, while during the twenty-four hours from noon till noon of the next day only 3 79 grams were eliminated in the urine It does not seem possible that the explanation of this is due to non-absorption of glutamic acid, for given with Liebig's extract it is freely absorbed, nor is it probable that it can be stored without change, for this result was obtained on the second consecutive day of its administration[2]

[2] The matter has not been followed to a conclusion, but it may be possible that glutamic acid favors synthetic construction of new protein

J 20 grams of alanine plus Portion I

In paper five it was shown that the ingestion of 20 grams of alanine with Liebig's extract of meat caused a rise of heat production from 16 2 calories to 19 2 calories per hour extending through the fifth hour To the same dog, Portion I with 20 grams of alanine was administered with the following results

Experiments 34 and 32

20 grams of alanine + Portion I at noon

TIME	PROTEIN N	AMINO ACID N	NON PROTEIN R Q	CALORIES				
				Protein	Amino-acid	Non-protein	Total Calculated	Total Found
1 00–2 00	0 212	0 047	0 96	5 63	0 91	12 03	18 57	21 19
2 00–3 00	0 225	0 169	0 86	5 98	3 27	11 29	20 54	20 84
3 00–4 00	0 209	0 242	1 05	5 55	4 67	9 12	19 34	21 68
4 00–5 00	0 197	0 220	0 83	5 21	4 25	11 07	20 53	21 08
5 00–6 00	0 187	0 136	0 92	4 95	2 62	13 92	21 49	22 76
Total				27 32	15 72	57 43	100 47	107 55
Per hour							20 09	

The experiments presented here should be compared with Experiments 25 and 27 when Portion I was given without the addition of alanine and in which the metabolism was 21 4 and 20 6 calories per hour *There is no increase in metabolism due to the addition of 20 grams of alanine to Portion I* In paper five, it was estimated that 15 46 calories were liberated from alanine metabolized in four hours after the administration of 20 grams of the substance in Liebig's broth In the present experiment, the estimated increase is 15 7 calories for five hours *Although alanine was certainly metabolized, it exerted no "specific dynamic" influence when given with the mixed diet which produced a total metabolism of 20 6 calories per hour, although administered alone it raised the metabolism from the basal value of 16 2 to 19 2 calories per hour*

The urinary analyses After adding 20 grams of alanine to Portion I, the urinary nitrogen nearly doubled in quantity although no such effect was obtained with glutamic acid The results are given below

Urinary analyses after giving Portion I plus 20 grams of alanine at noon

TIME	MARCH 1*		MARCH 4	
	TOTAL N	UREA + NH_3 N	TOTAL N	UREA + NH_3 N
12 00–1 00	0 234	0 181	0 260	0 218
1 00–2 00	0 234	0 181	0 260	0 174
2 00–3 00	0 396	0 289	0 466	0 369
3 00–4 00	0 396	0 289	0 519	0 409
4 00–5 00	0 460	0 369	0 484	0 379
5 00–6 00	0 460	0 369	0 338	0 295
Total	2 180	1 678	2 327	1 844
Per cent		77		79

* The urine of this day was obtained in periods of two hours each

On March 7, Experiment 32, and on March 9, Experiment 34, when the dog was in the calorimeter, the following urinary analyses were made and are compared with the figures obtained on March 4, as given above

DATE	TIME	TOTAL N	UREA + NH_3 N	PER CENT
March 7	12 00–6 00	2 564	1 995	78
March 4	12 00–6 00	2 327	1 844	79
March 9	12 00–4 00	1 397	0 971	70
March 4	12 00–4 00	1 505	1 170	79

The nitrogen figures found on March 4 appear, therefore, to be a trustworthy indication of the results on the days when the dog was in the calorimeter To determine the hourly protein metabolism, the nitrogen figures obtained when Portion I was given on March 19 were adopted To determine the metabolism of alanine, the difference between the urea plus ammonia nitrogen figures obtained on March 19 after giving Portion I and the urea plus ammonia nitrogen after giving Portion I plus alanine on March 4, was calculated, and with the following results

Time	N of alanine metabolized
12 00–1 00	0 122
1 00–2 00	0 047
2 00–3 00	0 169
3 00–4 00	0 242
4 00–5 00	0 220
5 00–6 00	0 136
Total	0 936

The figures were adopted in the hourly computations in Experiments 32 and 34 The objections to the method were discussed in paper five

K 20 grams of glycocoll plus Portion I

The results obtained after giving 25 grams of glycocoll were shown in paper five The metabolism rose from 16 2 calories to 21 8 and 22 8 calories per hour during the second to fifth hours after its ingestion In the following experiments, 20 grams of glycocoll were given with Portion I with the following results

Experiments 35 and 37

20 grams of glycocoll plus Portion I at noon

TIME	URINARY PROTEIN N	URINARY AMINO ACID N	NON PROTEIN R Q	CALORIES				
				Protein	Amino-acid	Non-protein	Total Calculated	Total Found
1 00–2 00	0 199	0 091	0 95	5 41	0 90	15 67	21 98	22 52
2 00–3 00	0 225	0 090	0 89	5 98	0 89	16 44	23 31	21 80
3 00–4 00	0 209	0 130	0 86	5 49	1 28	18 15	24 92	22 13
4 00–5 00	0 197	0 222	0 93	5 21	2 20	13 75	21 16	20 44
Total				22 09	5 27	64 01	91 37	86 89
Per hour							22 85	21 72
1 00–2 00	0 199	0 091	0 91	5 41	0 90	16 86	23 17	22 05
2 00–3 00	0 225	0 090	0 89	5 98	0 89	17 23	24 10	22 25
3 00–4 00	0 209	0 130	1 06	5 49	1 28	14 26	21 03	22 59
4 00–5 00	0 197	0 222	0 93	5 21	2 20	15 28	22 69	21 99
Total				22 09	5 27	63 63	90 99	88 88
Per hour							22 75	22 22

In order to interpret these experiments, comparisons are made below between the results obtained with (1) Portion I alone, (2) Portion I plus 20 grams of glycocoll and (3) 25 grams of glycocoll alone (see paper five)

ODY TEMPERATURE		MORNING WEIGHT	BEHAVIOR OF DOG	FOOD AT NOON
End	Difference			
		kg		
38 31	+0 11	9 4	Very quiet	100 grams meat
38 35	+0 04		Very quiet	100 grams biscuit meal
38 46	+0 11		Moving 10 min	20 grams lard
38 54	+0 08		Moving 10 min	
39 06	+0 52	8 9	Very quiet	100 grams meat
39 42	+0 36		Quiet	100 grams biscuit meal
39 55	+0 13		Moving 3 min	20 grams lard
37 85	+0 08	9 4	Very quiet	33 grams meat
38 05	+0 20		Very quiet	100 grams biscuit meal
38 24	+0 19		Action 2½ min	20 grams lard
38 08	+0 19	9 3	Very quiet	33 grams meat
38 22	+0 14		Very quiet	100 grams biscuit meal
38 34	+0 12		Moving 2 min	20 grams lard
38 32	−0 02		Very quiet	20 grams glutamic acid
38 67	+0 01	9 2	Very quiet	33 grams meat
38 78	+0 11		Moving 8 min	50 grams biscuit meal
38 86	+0 08		Very quiet	10 grams lard
38 84	−0 02		Very quiet	
38 82	+0 35	9 5	Very quiet	33 grams meat
38 96	+0 14		Quiet	50 grams biscuit meal
38 64	−0 32		Moving 1 min	10 grams lard
38 90	+0 26		Quiet	
38 80	−0 10		Moving 4 min	

Body temperature			Morning weight	Behavior of dog	Food at noon
Start	End	Difference			
			kg		
38 35	38 45	+0 10	9 4	Very quiet	50 grams biscuit meal
	38 52	+0 07		Very quiet	
	38 62	+0 10		Moving 2 min	
	38 60	−0 02		Moving 4 min	
38 02	38 22	+0 20	9 5	Quiet	50 grams biscuit meal
	38 46	+0 24		Moving 1 min	10 grams lard
	38 52	+0 06		Moving 2 min	
38 35	38 60	+0 25	9 4	Quiet	33 grams meat
	38 64	+0 04		Quiet	50 grams biscuit meal
	38 45	−0 19		Quiet	10 grams lard
	38 66	+0 21		Moving 2 min	20 grams glutamic acid
38 63	38 90	+0 27	9 4	Very quiet	33 grams meat
	38 82	−0 08		Quiet	50 grams biscuit meal
	38 96	+0 14		Moving 4 min	10 grams lard
					20 grams l-alanine
38 80	38 76	−0 04	9 4	Quiet	33 grams meat
	38 86	+0 10		Moving 1 min	50 grams biscuit meal
	39 00	+0 14		Moving 2 min	10 grams lard
					20 grams l-alanine
38 08	38 39	+0 31	9 5	Very quiet	33 grams meat
	38 29	−0 10		Very quiet	50 grams biscuit meal
	38 28	−0 01		Moving 4 min	10 grams lard
	38 11	+0 03		Moving 4 min	20 grams glycocoll
38 01	38 04	+0 03	9 5	Quiet	33 grams meat
	38 01	−0 03		Quiet	50 grams biscuit meal
	38 09	+0 08		Quiet	10 grams lard
	37 88	−0 21		Moving 1 min	20 grams glycocoll

appears in the urine of fasting men, rabbits and dogs but the amount present in the urine of the dog may be very little influenced by fasting [5]

Folin and Denis[6] have confirmed the observation of Rose respecting the presence of creatine in the urine of young children even when no creatine is taken in the food They attribute the presence of creatine to the high protein content of the children's diet

It is difficult to reconcile the fact that children excrete creatine with the theory of Mendel and Rose that creatine does not result from the metabolism of exogenous or of reserve protein, but only from endogenous metabolism Well-fed children take so much carbohydrate as to make one doubt whether a sufficient carbohydrate hunger could ensue (during the night) to account for the relatively high values regularly found for creatine

The present work was planned to throw light on whether creatine can originate from exogenous protein metabolism and, if so, what is the influence of the plane of protein intake on creatine

TABLE I

Creatine and creatinine elimination during inanition in the pig

Weight of pig, 85 pounds Urine preserved with a solution of thymol in toluene Starvation started January 19

DATE	VOLUME OF URINE	TOTAL N	CREATININE	TOTAL CREATININE
January	*cc*	*grams*	*gram*	*gram*
25	355	3 713	0 898	0 898
26	300	3 906	0 916	0 900
27	350	3 518	0 821	0 798
28	216	3 101	*	0 829
29	170	2 822	0 688	0 706
30	184	2 903	0 745	0 745
31	156	2 761	0 683	0 683
1	174	2 470	0 655	0 655
2	200	2 812	0 720	0 736

* Determination lost through the formation of difficultly soluble crystals on standing when preserved with picric acid in the volumetric flask

[5] Howe, Mattill and Hawk *loc cit*, Underhill and Kleiner this *Journal* iv, p 165, 1908, Mendel and Rose *ibid*, x, p 213, 1911, give a discussion of the literature of creatine and creatinine metabolism, Dorner *Zeitschr f physiol Chem*, lii, pp 239, 243, 1907

[6] Folin and Denis this *Journal*, xi, p 253, 1912

excretion when the energy metabolized remains constant Pigs were employed as experimental animals

At the outset we were surprised to find that moderate fasting does not lead to creatine elimination in the pig One of us has noted[7] that after a few days on a diet of starch and salts the urine of growing pigs becomes creatine-free We have conducted two experiments in which pigs were fasted for fourteen and sixteen days respectively without the appearance of appreciable amounts of creatine in the urine Table I gives the results of one of these experiments In the other case only the urines of the thirteenth to sixteenth days were examined and creatine was found to be absent

Of the different species of animals observed the rabbit seems to be the most sensitive to fasting, a high nitrogen and creatine output appearing in two or three days It is a significant fact that Mendel and Rose[8] obtained the largest creatine elimination with the most poorly nourished rabbit during starvation The dog is much less sensitive and from our observations it appears that the pig is still less so than the dog The fact that a well-nourished pig can fast from fourteen to sixteen days without excreting appreciable amounts of creatine, sheds considerable light on the interpretation which should be put upon the observations on this point made upon other species

The most plausible explanation of these results would seem to be the assumption of different degrees of ability to utilize fat as a source of energy When the rabbit fasts, the total nitrogen rises rapidly indicating an increase in endogenous nitrogen catabolism [9] This does not happen in the dog except possibly in a slight degree and our own observations on fasting pigs have shown that the total nitrogen in the urine steadily falls to a very low level On teleological grounds one might reason that, since the pig is one of the most efficient storers of fat, he might be expected to utilize it readily when necessary for energy production This seems to be the case The elimination of much creatine by the rabbit during fasting is unquestionably the result of increased tissue catabolism due to his inability to draw his supply of energy from other

[7] McCollum *Amer Journ of Physiol*, xxix, p 210, 1911

[8] This *Journal*, x, p 222, 1911

[9] Cf Mendel and Rose *ibid*

sources (fat) An endogenous origin of creatine seems therefore to be definitely established In the dog, fat is oxidized or otherwise transformed for energy production with much greater facility than in the rabbit and the endogenous metabolism is therefore not greatly accelerated during starvation so long as fat is available In the pig, since we find no creatine in the urine during the early stages of fasting (14–16 days), it would seem that fat is utilized so readily or energy production that no acceleration of tissue catabolism takes place The small amount of creatine of endogenous origin, when the latter type of catabolism is at its minimum, is destroyed in the body All observers agree that on a liberal carbohydrate diet no creatine is excreted by any of the species referred to above Our experience, in accord with others, shows that there exists considerable irregularity in the excretion of creatine on a uniform diet This has been attributed to a destruction of creatine by enzyme action We have data ourselves tending to substantiate this idea Urines preserved with both toluene and chloroform and incubated at 37° showed a regular and fairly rapid decrease in creatine and total creatinine content These enzymes, on the basis of the work of Gottlieb and Stangassinger[10] and of Rothman,[11] seem to be generally distributed through the animal tissues

TABLE II

Weight of pig, 66 pounds at beginning, 67 pounds at end of record Diet, 825 grams corn meal, 13 85 grams N and 2902 calories Calories per kilo, 97 (55 × creatinine N)

DATE	VOLUME OF URINE	N IN URINE	CREATININE	CREATINE	CREATINE AS PERCENTAGE OF TOTAL CREATININE
May	*cc*	*grams*	*gram*	*gram*	
25	923	6 770	0 7672	0 0326	4 07
26	1170	7 500	0 9266	0 0	0 0
27	1230	7 380	0 7140	0 0	0 0
28	1230	7 257	0 7683	0 0	0 0
29	1210	8 107	0 8615	0 0	0 0
30	1570	9 263	0 5893	0 0448	7 11
31	1410	7 896	0 8184	0 0093	1 12

[10] *Zeitschr f physiol Chem* , lii, p 1, 1907
[11] *Ibid* lvii, p 131, 1908

types can be synthesized in large amounts by birds taking rations free from this class of compounds

Experiments with fat-free rations compounded of isolated and purified food substances have not yet been successfully employed in experiments involving reproduction [8] In our own work we have employed a ration consisting of 30 per cent Merrill-Soule's skim milk powder and 70 per cent polished rice, the latter thoroughly extracted with boiling alcohol The milk powder contained 0 6 per cent of ether-soluble matter and the ether extract was practically free from phosphorus The lecithins present in the whole milk are nearly completely removed with the cream by the centrifugal process The rice, before extraction, was ground to a fine powder Two extractions were made, using a liberal amount of 95 per cent alcohol and maintaining a boiling temperature for from twenty to thirty minutes It was then transferred to a press and nearly freed from alcohol, and finally spread in a thin layer until the alcohol was all evaporated The rice thus treated was practically fat- and lecithin-free

The hens selected for the experiment were hatched in June 1910, and were fed an ordinary mixed ration until October 31 when they were placed in a cage, the floor of which was covered with clean sand The hens were kept in the attic of the poultry building throughout the experiment There was no failure of the appetite at any time The initial weights of the birds were as follows No 102, 967 grams, No 103, 1476 grams, No 106, 1184 grams

The average consumption of food per day from October 31, 1910, to March 1, 1911, was 175 6 grams for the three or 58 5 grams per day per bird The energy intake was about 235 calories per day for each hen The weights of the hens on January 30, at which date the first eggs were secured, were as follows No 102, 1290 grams, gain 33 per cent, No 103, 2013 grams, gain, 36 4 per cent, No 106, 1588 grams, gain, 34 1 per cent

The hens were not trap-nested so no individual records for egg production can be given Between January 30 and April 15, fifty-seven eggs were secured It is known that at least three eggs were eaten by the hens The egg production was therefore not far from normal for pullets of this age

[8] See Osborne and Mendel this *Journal*, xii, p 81, 1912

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[8] See Osborne and Mendel this *Journal*, xii, p 81, 1912

The yolks from the fifty-seven eggs weighed, as separated from the whites, 883 5 grams, an average yolk production of 294 5 grams per hen The average white production per hen was 465 5 grams As the eggs were collected they were weighed, broken and the whites and yolks separated and separately weighed The yolks were dropped into 95 per cent alcohol and preserved until the end of the experiment

A mixed sample of these yolks was found, by the method of Koch and Woods,[9] to contain 3 per cent of lecithin and 6 39 per cent of kephalin, a total phosphatide content of 9 39 per cent Each hen deposited in the egg-yolks produced by her 27 65 grams of phosphorized fats It is fair to assume that the bodies of the hens contained more phosphatides at the end of the experiment than at the beginning, and also that some phosphatides were present in the whites The fat content of the fresh yolks was 32 8 per cent

Since *the average daily consumption of skim milk powder* was 17 55 grams, the fat content of which was 0 105 gram, the fat intake per hen during the feeding period of one hundred and sixty-five days was 17 37 grams Being ordinary butter fat most of it was not suitable for direct deposition in the eggs It is evident therefore that the synthesis of phosphatides is readily accomplished in the body of the hen when the ration is free from these substances

We possess but little evidence as to whether the lecithins of the egg yolk are of a specific nature, or are of a variable character and influenced by the character of the fats or lecithins of the food If the lecithins of the yolk are transferred without change to the nervous system of the developing chick it would seem highly probable that the fatty acid radicals of the lecithins must be of a specific and unvarying character It is highly improbable that the composition of the nervous system of the chick, as respects the phosphatides, which form a part of the functioning organized matter, can vary through the substitution of, for example, oleyl-stearyl-lecithin, which might be abundant in the food, for oleyl-palmityl-lecithin, which the animal might synthesize if compelled to do so by lack of compounds of this character in the diet On the other

[9] Koch and Woods this *Journal*, i, p 203, 1905

hand, that portion of the lecithin which is broken down during incubation to supply phosphoric acid for skeletal formation might, so far as one can see, be equally efficient if made of any of the ordinary fatty acid radicals, since these all yield about the same amount of energy Mesernizky[10] has shown that the lecithin content of the egg decreases markedly during incubation

Bang[11] holds that since the proteins as we know them in the purified state are relatively stable substances, and we have no evidence that any high degree of lability exists in the proteins present in the living protoplasm, it is justifiable to attribute greater importance to the unstable complex lipoids which form a part of every living cell, than to the proteins, that the lipoids, among all the components of the colloidal systems which manifest the properties of living matter, are the most labile, and deserve therefore to be assigned an important rôle in the metabolic processes If this assumption be justified one would expect to find for a portion of the lecithins of the yolk a specificity of composition and structure This view is supported by the work of Henriques and Hansen[12] who conclude that the fatty acids of yolk lecithins are constant in character (iodine number) and independent of the character of the food lipoids, while the fats of the eggs are variable and easily influenced by the fats of the diet

It should be stated that in the experiments of Henriques and Hansen the periods of experimental feeding were shorter than thirty days in six periods and were forty-two days in the other two, and eggs were collected during the entire periods An effort was made to change by this method the nature of the fatty acid radicals of the egg lecithins by feeding barley, peas, rice, hempseed and linseed, and iodine numbers of 72 5, 70 8, 66 9, 74 7 and 78 4 respectively were obtained for the lecithins There is no evidence that in their experiments a synthesis of lecithins actually occurred

Of still greater interest is their observation that the iodine numbers of all lecithins examined by them were such as to indicate for the fatty acids themselves an iodine number of about 100, which would indicate the presence of acids having a higher iodine number than oleic acid (80–90) The idea of a specificity of the

[10] Mesernizky *Biochem Centralbl* , vi, p 784, 1907
[11] Bang *Ergeb d Physiol* , vi, p 134, 1907
[12] Henriques and Hansen *Skand Arch f Physiol* , xiv, p 390, 1903

character of the lecithins is strengthened by their observation that preparations of lecithins from dog and ox brains showed nearly the same iodine numbers (96–100) as did those from egg lecithins

These results of Henriques and Hansen make especially interesting the iodine numbers of lecithins produced by our hens where a complete synthesis of all the organic complexes of the lecithins was made absolutely certain The following table gives the values for the fats and lecithins of the eggs produced in the experiments here described, and, for comparison, the corresponding values for eggs from the farm flock on an ordinary ration

	IODINE NUMBER		
	I	II	III
Fats			
Nearly lipoid-free ration	50 0	54 36	51 1
Ordinary ration	63 2	65 5	
Lecithins			
Nearly lipoid-free ration	35 22	34 07	34 0
Ordinary ration	63 7	63 1	

The method of obtaining the lecithins was as follows

The yolks were removed from the alcohol and placed in an evaporating dish, covered with a watch glass and heated on a steam bath for a short time to remove most of the alcohol Ether was then added repeatedly in small portions and the evaporation continued until the alcohol was all removed By this process the yolks were protected as much as possible from the air The yolks were then dried in a desiccator over sulphuric acid, the desiccator being evacuated by the ether method When dry, the sample was transferred to an extraction apparatus and thoroughly extracted with ether The ether was evaporated, the last part being removed over sulphuric acid in a desiccator Great care was taken to prevent exposure of the fat and lecithin preparations to the air The residue from the ether extraction was extracted with seven or eight portions of hot alcohol (95 per cent), each digestion being continued about fifteen minutes The combined alcohol extracts were concentrated on a steam bath Toward the end of the evaporation small portions of ether were repeatedly added to hasten the removal of the alcohol and to protect the lipoids from the air The product was finally dissolved in ether and precipitated with ten volumes of acetone After once reprecipitating, the lecithins were dried in a vacuum desiccator over sulphuric acid The alcohol in which the eggs were preserved was evaporated in the manner described and the residue treated in the same way as the yolks

The iodine numbers were made on different samples and there is no doubt that they represent the true values for the fats and lecithins from the mixed eggs From these results we must conclude that the lecithins of the egg yolk are variable in respect to the nature of the fatty acid radicals they contain and that their nature is influenced by the character of the lipoids of the diet The iodine numbers of lecithins in yolks from a nearly lipoid-free diet correspond fairly well with those which one might expect for a lecithin containing one molecule of oleic and one of a saturated fatty acid It is significant that the lowest iodine number obtained by Henriques and Hansen was from a rice diet which was very poor in lipoids

Unfortunately none of the eggs with these low iodine numbers were hatched, but this will be done as soon as more can be produced It will be of interest to compare the lecithins of the nervous system of chicks, hatched from such eggs, with those of chicks obtained after feeding rations similar to those of Henriques and Hansen, when the rations are fed long enough to secure the maximum influence of an abundance of highly unsaturated lipoids of the diet on the lecithins of the egg

METABOLISM STUDIES ON COLD-BLOODED ANIMALS I

THE URINE OF THE FISH

By W DENIS

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(Received for publication, September 24, 1912)

Probably on account of the difficulties attendant on their collection the data at hand regarding the composition of the urine of marine animals are extremely limited, and decidedly fragmentary in character, in fact so far as the author is able to ascertain, no analyses showing the distribution of nitrogen in twenty-four-hour quantities of urine in even the larger marine animals, such as the fish, are to be found in the literature

As, by means of the micro-chemical methods for the analysis of urine recently published by Folin and his associates, it is now possible to make observations on the distribution of nitrogen in twenty-four-hour samples of the urine of relatively small animals, the time seemed ripe for an effort to investigate the metabolism of lower animals, from the standpoint of urine analysis, more thoroughly than has so far been attempted

As the great majority of fish possess no bladder but allow the urine to escape as soon as formed, it was first necessary to find some method by which this fluid could be collected accurately over twenty-four-hour periods My original intention was to study the urine of both the elasmobranchs and of the teleosts, it was found impossible, however, in the period during which the work of collection was carried on to obtain enough specimens of any one species of bony fish to make work along this line profitable, in the present paper, therefore, the data presented consist largely of observations on the urine of one of the elasmobranchs, the smooth dog fish (*Mustelis canis*), which is extraordinarily abundant along the north Atlantic coast Analytical data concerning the urine of

one bony fish, the goose fish (*Lophius piscatorius*), are also presented, the material for this work having been taken from the bladder shortly after death

The method used for the collection of twenty-four-hour samples of urine from the dog fish was as follows [1]

Large male animals were used almost entirely The female may be employed but in the male the insertion of the cannula is accomplished with much greater ease than in the female The animal was first securely fastened, dorsal surface up, in a holder consisting of a V-shaped trough about 18 inches long and 6 inches deep The fish fastened to the holder was then placed in a long narrow tank of running sea water in such a position that while the head including the gills was under water the remaining two-thirds of the body including the cloaca was above the surface of the liquid

The cloaca was then stretched open by means of hooks inserted laterally and an ordinary glass cannula inserted and securely tied directly into the urinary papilla The latter operation is particularly easy to accomplish in the mature male in which the papilla may be as much as a quarter of an inch in length In the male the cannula may now be tied by means of thread to the "claspers" In the female it is necessary to suture it to the fins in order that it may not be pulled out during the subsequent struggles of the fish

The animal was now removed from its holder, placed on its side and carefully tied by means of inch wide bandages to a board about 2 feet in length The whole was now placed in a large tank, well supplied with running sea water, and was fastened in such a position that the cloaca was raised above the surface of the liquid A wet towel placed over the tail kept the latter moist

The urine was collected in a large test tube which was hung on the side of the tank considerably below the head of the fish This test tube was closed by means of a two-hole rubber stopper, through one hole of which passed a short glass tube, which in turn connected with a piece of narrow pure gum tubing, the other end of which was attached to the cannula in the urinary papilla

All urine samples were preserved with chloroform and were analyzed for nitrogenous constituents not later than thirty hours after collection

The method outlined above is extremely simple and has the advantage that as no surgical procedure is necessary no anaesthetics need be used and at the same time elimination from the intestine is not interfered with Gentle pressure applied in the

[1] The method outlined above for the collection of urine was suggested to me by Mr Scott of the Department of Natural History of the College of the City of New York I have also to thank Mr Scott for much valuable assistance during the development of the method

median line of the dorsal surface about 1 to 6 inches above the cloaca will serve to expel any urine present in the ureters and, if this procedure be employed just before insertion and just previous to removal of the cannula, a fairly accurate measure of the amount of urine excreted in a given period of time may be obtained

If it is not desired to keep the fish bound to a board during the collection it may be conveniently placed in a trough so narrow that the animal is unable to turn around, I have used this method in a few cases but lack of conveniently arranged tanks made its exclusive adoption impossible

The method described above differs fundamentally from the procedures used by Herter,[2] by Baglioni[3] and by Burian[4] for the collection of fish urine in the fact that I have always arranged the cannula so that the urine was drained away as soon as formed The quantity of urine eliminated in twenty-four hours by the dog fish is surprisingly small considering the size of the animal, as will be shown by the results tabulated below All animals used were males

FISH NO	LENGTH	WEIGHT	VOLUME OF URINE COLLECTED IN TWENTY FOUR HOURS
	cm	*grams*	*cc*
26	94	2268	15
29	76	1134	11
30	86	1814	27
31	71	964	17
32	92	2041	30
35	69	964	25
36	81	1588	22
37	81	1360	27

The dog fish survives pithing of the cord remarkably well and will usually live apparently in good condition for several days after the operation As in many physiological experiments involving the collection of urine destruction of the cord is to be desired, I have made a number of collections of twenty-four-hour quantities of urine from fish in which the cord had been destroyed up to about the level of the dorsal fin

[2] Herter *Mitth aus der zool Stat zu Neapel*, x, p 342, 1891
[3] Burian *Zeitschr f biol Technik u Method*, i, p 383, 1909
[4] Baglioni *Zentralbl f Physiol*, xix, p 385, 1903

FISH NO	SEX	LENGTH	WEIGHT	VOLUME OF URINE COLLECTED IN TWENTY FOUR HOURS
		cm	grams	cc
1	♀	92	1815	20.0
3	♀	94	2722	21.0
9	♂	81	1446	19.5
11	♀	96	1810	13.0
18	♀	96	1588	20.0
19	♂	107	2948	44.0
20	♂	79	1174	17.0
21	♂	91	1813	17.5

As will be seen by an inspection of these results the quantity of urine eliminated in twenty-four hours is about the same in the pithed as in the normal animal, the average twenty-four-hour quantity for the former being 21.5 cc. and for the latter, 21.7 cc. An examination of the analytical results given below will show, however, that in the pithed animal a much less concentrated urine is secreted than in the normal fish.

When first passed, the urine of the dog fish is a clear odorless, almost colorless fluid with a slight yellowish-green tinge and a reaction distinctly acid to litmus. After standing for a few days, even when preserved with chloroform, it becomes cloudy, and the color darkens gradually until when several weeks old it possesses the color of a moderately concentrated human urine.

In all, the urine of about thirty animals was examined and in no case was any reducing sugar of albumin found. The murexide reaction is readily obtained. Creatinine appears to be present, but the amount is so small that quantitative measurements were impossible, even by the Folin method. No creatine could be detected.

The analytical methods used were as follows: specific gravity by the hydrometer, total nitrogen by the method of Folin and Farmer,[5] urea by the potassium acetate method of Folin,[6] ammonia and uric acid by the methods of Folin and Macallum,[7] phosphates

[5] This *Journal*, xi, p. 493, 1912.

[6] *Ibid.*, xi, p. 507, 1912.

[7] *Ibid.*, xi, pp. 265, 523, 1912. (The method used for the colorimetric determination of uric acid will be published shortly from this laboratory.)

by the uranium acetate titration and total sulphur by the author's[8] modification of the Benedict method The animals from which urine was collected had in every case been caught from twenty-four to forty-eight hours before the experiment was started, and during this time were kept in cages immersed in the ocean

Fish 30

Male, weight, 1814 grams, fasting, volume of urine collected in twenty-four hours, 27 cc , specific gravity, 1 032

	MILLIGRAMS IN TWENTY FOUR HOURS	MILLIGRAMS PER CC	PER CENT OF TOTAL NITROGEN
Total nitrogen	122 5	4 5	
Urea nitrogen	108 4	4 0	89 0
Ammonia nitrogen	2 3	0 085	1 9
Uric acid nitrogen	0 31	0 011	0 25
Creatinine nitrogen	Present		

Fish 29

Male, weight, 1134 grams, fasting, volume of urine collected in twenty-four hours, 11 cc

	MILLIGRAMS IN TWENTY FOUR HOURS	MILLIGRAMS PER CC	PER CENT OF TOTAL NITROGEN
Total nitrogen	58 3	5 3	
Urea nitrogen	51 5	4 7	88 6
Ammonia nitrogen	1 32	0 12	2 2

Fish 31

Male, weight, 964 grams, volume of urine collected in twenty-four hours, 17 cc This animal was allowed to feed on a large amount of fish muscle about six hours before the collection of urine was commenced

	MILLIGRAMS IN TWENTY FOUR HOURS	MILLIGRAMS PER CC	PER CENT OF TOTAL NITROGEN
Total nitrogen	113 0	6 6	
Urea nitrogen	93 4	5 5	82 6
Ammonia nitrogen	5 18	0 30	4 5

[8] This *Journal,* viii, p 401, 1910

Fish 19

Male, weight, 2948 grams, fasting, spinal cord pithed from tip of tail to about the level of the dorsal fin, volume of urine collected in twenty four hours, 44 cc

	MILLIGRAMS IN TWENTY FOUR HOURS	MILLIGRAMS PER CC	PER CENT OF TOTAL NITROGEN
Total nitrogen	104 7	2 37	
Urea nitrogen	88 2	2 00	84 2
Ammonia nitrogen	3 3	0 075	3 1
Creatinine nitrogen	Present		
Creatine nitrogen	None		
Uric acid nitrogen	0 56	0 012	0 5

Fish 19a

Male, weight, 2940 grams, fasting, spinal cord pithed, volume of urine collected in twenty-four hours, 27 cc

	MILLIGRAMS IN TWENTY FOUR HOURS	MILLIGRAMS PER CC	PER CENT OF TOTAL NITROGEN
Total nitrogen	62 00	2 3	
Urea nitrogen	53 00	1 96	85 2
Ammonia nitrogen	3 78	0 14	6 1
Uric acid nitrogen	0 40	0 015	0 64
Creatinine	Present		

Mixed urines of fishes 20 and 21, both males, weight of fish 20, 1174 grams, volume of urine for twenty-four hours, 17 cc, weight of fish 21, 1813 grams, volume of urine for twenty-four hours, 17 cc, both fasting, spinal cord pithed in both

	MILLIGRAMS PER 1000 CC	PER CENT OF TOTAL NITROGEN
Total nitrogen	3200	
Urea nitrogen	2600	81 0
Ammonia nitrogen	340	10 6
Creatinine nitrogen	Present	
Creatine nitrogen	None	
Uric acid nitrogen	22	0 68

Analysis of a composite sample of urine collected from ten fasting dog fish

Specific gravity, 1 030

	GRAMS PER LITER	PER CENT OF TOTAL NITROGEN
Total nitrogen	4 200	
Urea nitrogen	3 390	80 7
Ammonia nitrogen	0 310	7 3
Chlorides (as NaCl)	12 86	
Phosphates (as P_2O_5)	4 520	
Total sulphur (as SO_3)	7 08	
Total sulphates (as SO_3)	3 40	

The goose fish (*Lophius piscatorius*) proved of all the teleosts available to be the best suited for the study of the urinary secretion, as this animal is provided with a bladder of large capacity However, during the time spent at Woods Hole only one specimen was available in which the bladder contained urine In this case 158 cc of urine were removed from the bladder about an hour after death and immediately analyzed The urine of the goose fish is a clear, odorless, almost colorless fluid, which after long standing (when preserved with chloroform) becomes faintly yellow The specific gravity was found to be 1 013 The reaction is distinctly acid to litmus No albumin or reducing sugar could be detected As in the urine of the dog fish the amount of phosphates is so large that when the urine is boiled a thick flocculent precipitate is obtained which readily dissolves on the addition of a drop of acetic acid On making alkaline with sodium hydrate a large amount of precipitate is also obtained probably due to the presence of an excess of earthy phosphate

Uric acid is apparently not present as the murexide test gave negative results, while even with the very delicate phosphotungstic acid reagent[9] (which gives unmistakable results with 1 part of uric acid in 500,000 parts of water), used in the colorimetric method for the quantitative determination of the substance, no indication of its presence could be found [10]

[9] Folin and Denis this *Journal*, xii, p 237, 1912

[10] This is in agreement with the observations of Rywosch on the urine of the carp *Wien med Wochenschr* , 1893, pp 47, 48, and with the earlier work of Davy *Trans Roy Soc Edinburgh*, xxi, p 543, 857

No creatine or creatinine could be detected by the use of the Jaffé test

	MILLIGRAMS PER LITER	PER CENT OF TOTAL NITROGEN
Total nitrogen	400	
Urea nitrogen	248	62
Ammonia nitrogen	52	13

It is planned to extend this investigation to a study of the metabolism of a number of the cold-blooded animals, such as the fish, and of such of the amphibia and reptiles as may be found available for this type of work

MAINTENANCE EXPERIMENTS WITH ISOLATED PROTEINS[1]

By THOMAS B OSBORNE AND LAFAYETTE B MENDEL

WITH THE COÖPERATION OF EDNA L FERRY

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(Received for publication, September 26, 1912)

In earlier papers on our feeding experiments with isolated food substances[2] we have attempted to justify the selection of the white rat for the study of some of the problems connected with nutrition This animal is easily reared and cared for Its small size reduces the food requirement to a magnitude which falls within the range of experimental possibility where the preparation of special dietaries by laborious processes is a fundamental prerequisite A possible advantage in the use of smaller animals like those which we have selected lies in the fact that marked changes in nutritive equilibrium speedily manifest themselves Furthermore, the longevity of this animal is, according to Donaldson, about three years, so that the first year of life corresponds to a long span in terms of human years Not insignificant is the additional fact that the white rat has in recent years been made the subject of exceptionally extensive measurements in respect to growth and various features of development at the Wistar Institute in Philadelphia and elsewhere In this way physical standards, so to speak, have been established for this animal Inasmuch as we have successfully maintained albino rats over periods of more

[1] The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D C

[2] Osborne and Mendel Feeding Experiments with Isolated Food Substances Carnegie Institution of Washington, Publication 156, Parts I and II, 1911, *Zeitschr f physiol Chem* , lxxx, p 307, 1912, this *Journal*, xii, p 473, 1912

than 600 days on artificially prepared food mixtures we believe that the adaptability of the animal for the purposes under consideration must be admitted The chief obstacles to success have been those diseases, usually involving the lungs, which other workers with rats have also found troublesome These diseases, however, have made less inroad upon our carefully selected and cared for experimental animals than upon the animals of our stock colony We believe that by the application of further hygienic precaution the disease factor can be largely eliminated

The work of earlier investigators, who have attempted to determine whether any single protein or combination of proteins is capable of supplying the nitrogenous needs of the body, has been reviewed in an earlier publication[3] If we except the investigations of Rohmann,[4] the details of which have not yet been published, it would appear from the records of our predecessors that "experiments in which one form of protein has been given as the sole source of nitrogen for a long period demonstrate that, in spite of the abundance of nitrogen in the diet, the animal ceases to thrive[5] Experiments in which the foodstuffs administered were sufficiently "pure" to give some permanent significance to the results obtained are few in number Occasional records show that animals have been kept over one hundred days, as a rule, however, part of this period has been characterized by a decline in body weight which obviously must vitiate the success of any demonstration of maintenance on "artificial" diets The reserve store of nutrients in many animals is sufficient to keep them alive for considerable periods even on inadequate dietaries, so that the continuance of life is by no means equivalent to adequate nutritive maintenance This fact has too often been overlooked in the interpretation of nutrition experiments

The futility of studying nutrition by methods in which even the "control" animals decline ought to be obvious and is brought to mind by recent experiments of Frank and Schittenhelm[6] who failed

[3] Osborne and Mendel, Carnegie Institution of Washington, Publication 156, Part I, p 6, 1911, see also Cathcart *The Physiology of Protein Metabolism*, 1912, p 74

[4] Röhmann *Biochem Zeitschr*, xxxix, p 507, 1912

[5] Cathcart *The Physiology of Protein Metabolism*, 1912, p 74

[6] Frank and Schittenhelm *Therapeutische Monatshefte*, xxvi, p 112, 1912

to nourish rats adequately on a diet of egg-white, starch, glucose, fat, cellulose and salt mixture Their rats declined within thirty days on this diet which was selected to be compared with a dietary in which completely digested proteins formed the source of nitrogen The authors emphasize the fact that such digestion products may at times contain objectionable toxic substances (amines?) Their insistence on the use of "pure" proteins, however, loses part of its significance in the light of the fact that none of their artificial feedings were reasonably successful even with supposedly favorable selections of diet

In explanation of the failures of our predecessors various suggestions have been offered The failure to eat sufficient food has clearly been a frequent obstacle, as has been emphasized among others by McCollum [7] Aside from this, however, "much of the earlier work in this connection," as Cathcart remarks, "was faulty owing either to the manner in which the experiments were carried out, or to the fact that the diets could not be regarded as 'pure,' *i e*, the protein used was not absolutely free from impurities " He adds "Notwithstanding this it has been found that if the animals be kept for a prolonged period on one diet they invariably die in spite of an abundant caloric intake [8] We propose to consider this criticism carefully in the experiments to be reported below

The necessity of long continued experiments calls for particular emphasis Physiological alterations dependent upon the gradual depletion of a small store of essential tissue material may manifest themselves with extreme slowness, and the fact that a satisfactory nutritive balance can be maintained for a week or two or even a month in some cases is no guarantee of either the ultimate success of the dietary or of the impossibility of a decline owing to the inappropriate exhibition of an essential ingredient (cf Charts 1, 2 and 3) This has further been brought out most strikingly in the splendid study of Hart, McCollum Steenbock and Humphrey on the physiological effects on growth and reproduction of rations balanced from restricted sources [9] They have shown that animals fed rations from different plant sources and comparably balanced

[7] McCollum *Amer Journ of Physiol*, xxv, p 120, 1909

[8] Cathcart *The Physiology of Protein Metabolism*, 1912, p 74

[9] Hart, McCollum, Steenbock and Humphrey University of Wisconsin Agricultural Experiment Station, Research Bulletin No 17, 1911

in regard to the supply of digestible organic nutrients and production therms were not alike in respect to general vigor, size and strength of offspring and capacity for milk secretion The records extend over three years so that the gradual manifestations of departure from the normal standards of health and physiological efficiency which failed to reveal themselves in the earlier period were not overlooked As these authors say, "unquestionably the physiological value of a ration is largely dependent upon its chemical constituents, but the usual determinations made on feeding materials do not reveal the character or manner of combination of many of the constituents Consequently the physiological value can be determined, in the present state of our knowledge, only by long continued observations of the reaction of the feed on the animal "

A consideration of the prolonged maintenance of any animal raises the question as to its natural duration of life In the case of the white rat we know of few statements which can lay claim to any accurate basis The current view, based apparently on a statement by Donaldson, that the average life span of the albino rat is about three years, rests on the fact that there had been no records of a survival of this period in animals under observation Recent studies by Slonaker[10] indicate that a white rat can live to an age of 1361 days The incidence of disease in a large rat colony must be taken into account in connection with duration tests, and it has proved to be less detrimental among our isolated experimental animals than in the larger colony where the animals are maintained on mixed food under conditions more nearly like those to which they are accustomed by nature In fact the mortality among our experimental rats has thus far been smaller than among our supply animals

The weight of the animals constitutes, we believe, the best index at the present time of satisfactory nutritive maintenance The necessity of distinguishing carefully between nutrition in maintenance and in growth has been emphasized in another place [11]

[10] Slonaker *Journal of Animal Behavior*, ii, pp 20–42, 1912, also The Effect of a Strictly Vegetable Diet on the Spontaneous Activity, the Rate of Growth, and the Longevity of the Albino Rat, *Leland Stanford Junior University Publications*, 1912

[11] Osborne and Mendel *Zeitschr f physiol Chem* lxxx, p 307, 1912

Perhaps, as Waters has suggested, the term *maintenance* has been used somewhat loosely in the past, but like others we have been in the habit of regarding the animal in maintenance when its live weight was constant "A more correct definition of the term would perhaps be to say that an animal was in maintenance when its body was in energy balance, but the live weight has been the conventional measure of our maintenance values "[12] In the present report we are concerned solely with the maintenance features

Under selected conditions of diet an animal can be maintained adequately without growth (cf Chart 4) Furthermore a reparation of tissue is not necessarily identical with growth (cf the weight gained by Rat 134, in Chart 5, days 72 to 120, which shows that it is possible to restore tissue loss, exemplified in decline of body weight, by the use of dietaries which are inadequate for growth) Recovery from the decline due to malnutrition, for example, may thus be brought about by gliadin feeding The possible dissimilarity of the processes of maintenance, growth, and repair, in so far as they affect the rôle of proteins in nutrition, has also been emphasized by McCollum [13] The distinctions here made are illustrated by many of the charts in the appendix

It is perhaps unnecessary to remark that there may be involved in the problems of maintenance and growth many other factors, viz , the total energy intake, the character of the inorganic salts, the specific nature of the carbohydrates in the diet, quantitative and qualitative differences[14] in the proteins administered as well as the indefinable so-called "hormones" Indeed some writers at the present time believe that in the latter, as yet unknown factors, rests the secret to nutritive success Thus Cathcart has lately written, "it is clear that apart from the caloric intake and the protein, carbohydrate and fat content of the food, there is some factor or factors which influences the utilization, perhaps

[12] Waters The Capacity of Animals to grow under Adverse Conditions, *Proceedings Society for the Promotion of Agricultural Science*, xxix, p 3, 1908

[13] McCollum *Amer Journ of Physiol* , xxix, p 215, 1911

[14] The choice of a protein content of about 18 per cent in our food mixtures has been determined by nutrition trials with varying concentrations of protein in the diet These will be described elsewhere The proportions here selected fall within the range of our most successful experimen [illegible] n the non-protein factors are otherwise the same

also the amount of food required. This evidence practically points to the presence of some 'mineral' substance, or substances, in normal food which are absent in 'pure' food."[15] In an earlier report it was stated that an animal had been fed more than 217 days on a diet in which the sole protein was glutenin. The complete record of this animal, Rat 71, is given in Chart 6, since it illustrates a number of important features connected with our feeding experiments. It will be noted that the decline after 300 days of experimental feeding which could not be prevented by the addition of a second protein, edestin, to the diet was speedily prevented by the administration of mixed food during a period of one week. When the earlier glutenin food was again fed in period 8 a gradual decline again set in; this was however promptly averted, and striking reparation made, when "protein-free milk" was used to replace the inorganic salts and part of the carbohydrates of the dietary. On this food mixture the animal continued for 144 days until death ensued after 531 days of experimental feeding, the immediate cause being an abscess of the jaw resulting in the inability of the animal to eat. Old age may have contributed materially to the final decline of this rat. A peculiar significance centers about period 6 on the curve because it clearly shows that the factors determining successful nutrition were here involved in some other component of the dietary than the protein itself. The reasons which have led to the use of the protein-free milk as an adjuvant of our dietaries, as well as the possible criticisms in respect to a minimal protein content which may attach to its use, have been presented in detail elsewhere.[16]

We have already called attention to the necessity of long continued experiments if the actual sufficiency of a diet is to be determined. A deficiency in some essential ingredient may not make itself manifest for a long time, and even then be difficult to detect, for many other causes than defects in the food may lead to the decline or death of animals during experiments lasting over many months. It is only when the failure to be maintained is

[15] Cathcart: *The Physiology of Protein Metabolism*, 1912, p. 76; cf. also Hopkins: *Journ. of Physiol.*, xliv, p. 425, 1912; Suzuki, Shimamura and Odake: *Biochem. Zeitschr.*, xliii, p. 89, 1912.

[16] Osborne and Mendel: Carnegie Institution of Washington, Publication 156, Part II, 1911; *Zeitschr. f. physiol. Chem.*, lxxx, p. 307, 1912; This *Journal*, xii, p. 483, 1912.

the invariable outcome of the prolonged feeding on a given diet that we are justified in assuming that the diet is in some way inadequate, and then only when a prompt and complete recovery ensues when the diet is changed to one that is known to be in all respects sufficient (cf Chart 1, period 3, Chart 2, period 4, Chart 3, period 5, and Chart 6, period 6) Our experience shows that every animal has sooner or later declined when fed with mixtures of isolated and purified proteins, carbohydrates and fats together with inorganic matter in the form of crystallized salts In nearly every case the decline has been sudden, with strong evidence that death would soon have ensued had not the food been changed In each case immediate recovery has followed a change in the diet, thus showing the experimental foods to be inadequate for prolonged nutrition

Whether the deficiency of the purely artificial diet is to be attributed to improper proportions of its constituents, to improper combinations of these constituents, or to the lack of some essential element, is at present difficult to determine That the elements essential for prolonged maintenance are present in milk from which the fat and protein have been removed is also shown in the charts already referred to In every case the substitution of a food containing its inorganic constituents and a part of its carbohydrate in the form of this so-called "protein-free milk" has resulted in immediate recovery of the depleted animal, and thereafter the animal has continued in a well-nourished state until its life terminated from disease or old age If we compare the body weight curves of mature animals maintained in nutritive equilibrium on the purely artificial diet with those of animals fed on a diet known to be deficient in some element supposed to be essential for maintenance, we find a marked difference Thus animals kept on a diet free from inorganic salts or on one containing as its sole protein either zein or gelatin, which lack tryptophane, immediately decline and continue to fall in weight throughout the entire time of such feeding, whereas in the experiments with the purely artificial mixtures containing adequate proteins and inorganic salts a decline in weight occurs only after some time, and is then, in nearly every case, sudden and severe That no serious damage has been done to the animal is shown by its rapid recovery when protein-free milk is added to the food, however, that it has suffered in some way is indicated by the fact that when restored to its

original weight it cannot then be again maintained for any considerable time when returned to the original diet

Whether or not this means that its cells have lost something essential for their normal activity, which is furnished by the "protein-free milk," cannot now be determined, but the experimental evidence is sufficient to justify a search for such a substance in milk Recently published investigations by Hopkins[17] indicate that milk, as well as other natural food materials, contains a substance or substances which, even in very small quantities, suffices to induce normal and continued growth, for several weeks at least, in rats maintained on artificial mixtures of food substances which are otherwise inadequate for growth [18]

Charts 5, 7, 8, 9, 10 and 11 show that with the aid of "protein-free milk" *it is possible to maintain rats for periods equal to practically their entire adult lives on foods containing a single purified protein*, and also that the successful food proteins may differ very widely in their chemical make-up without affecting the physical well being of the animal to any noticeable extent If we consider that after hydrolysis with acids gliadin yields 25 per cent of its nitrogen as ammonia, while casein and edestin yield only 10 per cent, and that gliadin yields only 5 8 per cent of its nitrogen as basic amino-acids while casein yields 22 2 per cent and edestin 31 4 per cent, it is evident that the form in which the nitrogen is presented to the animal is very different for each of these proteins These differences are further emphasized by the proportion of some of the amino-acids determined by methods which give results of sufficient accuracy to justify comparison

	CASEIN	EDESTIN	GLIADIN
Arginine	3 81	14 17	3 16
Histidine	2 50	2 19	1 56
Lysine	5 95	1 65	*
Glutaminic acid	15 55	18 74	43 66
Glycocoll	0 00	3 80	0 00

* Regarding the presence of lysine in gliadin see Osborne and Mendel This *Journal* xii p 480 1912

[17] Hopkins *Journ of Physiol*, xliv, p 425, 1912

[18] Cf also Suzuki, Shimamura and Odake *Biochem Zeitschr*, xliii, p 89, 1912

Another important difference, which has usually been overlooked when comparing casein with other proteins, is that only about 0 1 per cent of sulphur can be obtained as sulphide from casein, whereas from edestin 0 35 and from gliadin 0 62 per cent can be obtained, thus showing that casein contains very little cystine

The possibility of successful maintenance is not by any means restricted to the particular proteins employed in these experiments It happens, however, that our longest records involve those proteins which earliest elicited our experimental interest, and which could be prepared in pure form most advantageously

Special importance centers in the remarkably successful maintenance of rats upon gliadin The continuance of Rat 130, for example (see Chart 11), during more than 530 days of adult life on a mixture of isolated food substances containing a single protein deficient in two familiar Bausteine, lysine and glycocoll,[19] and which affords an inadequate diet for satisfactory growth, furnishes by far the longest experiment on record of "artificial" nutrition and should serve to justify the renewal of studies with the isolated foodstuffs We have at present records of twelve rats which have been maintained more than 400 days on comparable food mixtures and five animals whose maintenance record exceeds 500 days These prolonged nutrition trials exceed in duration the best maintenance records which we have thus far obtained in our stock colony of animals fed on mixed foods Bearing in mind that these diets are, in addition to their probable freedom from glycocoll in the case of casein, and their deficiency in glycocoll and lysine in the case of gliadin, devoid of more than the merest possible traces of purines and of phosphoproteins, except in the case of casein, the synthetic activities of the organism are again clearly brought to mind It would surely be an extreme exaggeration of every metabolic probability to assume that in periods extending far longer than a year, *i e*, approximately one-half of an animal's span of life, the organism had conserved its store of the missing chemical complexes or altered its chemical make-up The latter assumption indeed is completely at variance both with the available evidence regarding the chemical

[19] Regarding the possible presence of lysine and glycocoll in gliadin, see Osborne and Mendel This *Journal*, xii, p 480, 1912

fixity of the tissues and biological considerations respecting the identity of species

The possible criticism which may be evoked by the use of "protein-free milk" containing a minute trace of unremoved milk protein (equivalent to about 0 5 per cent of the entire food), can be met satisfactorily only by maintenance experiments which we are now conducting with artificial mixtures of inorganic salts and carbohydrates [20] The trials have as yet continued over too short periods to be in any way comparable with the longer records above A few charts are, however, appended (see Charts 12 and 13) because they already indicate a considerable degree of success in the absence of milk protein as well as of the hypothetical organic "hormones," etc , present in the milk The experiences with the artificial salt mixture I (see Charts 2, 7, 10 and 14) afford evidence on this point, for in these experiments the animals were maintained in good health for 190, 360, 400 and 200 days respectively before declining in weight

As a corollary, so to speak, to the preceding experiments in successful maintenance are appended a few records of failures of maintenance associated with the administration of manifestly inadequate proteins (cf Charts 15, 16, 17, 18, 19, 20 and 21) In several of these the ready restoration of body weight by the addition of, or replacement by, adequate protein is clearly indicated It is interesting to note the more or less successful repair of nutritive failure by gliadin (see Charts 17, 18 and 21) which is inadequate to promote true growth [21]

The failure to be maintained on the inadequate proteins, zein and gelatin, is not to be ascribed to a failure in utilization, for nitrogen determinations made on the feces showed that the utilization of the protein was good

DISCUSSION

The currently discussed theories of metabolism emphasize the importance of the "Bausteine" as fundamental factors in protein metabolism, and they lay stress by inference, if not by experiment,

[20] Osborne and Mendel *Proc Soc for Exp Biol and Med* , ix, p 72, 1912

[21] The physiological role of gliadin is discussed by Osborne and Mendel This *Journal*, xii, p 473, 1912

upon the results of supplying these in adequate proportions as a primary requisite[22] Now and then there has arisen a protest against the general view that the similarity of the molecule of the food protein to that of the specific body proteins determines its relative food value to the animal, and that any one of the essential cleavage products which is present in smallest amount in the food protein determines the value of the entire molecule to the animal McCollum,[23] for example, has presented experimental data which do not harmonize entirely with the most widely accepted theories concerning the chemistry of protein metabolism and which he interprets to indicate that the processes of cellular catabolism and repair do not involve the construction and resynthesis of the entire protein molecule Abderhalden has lately summarized his view of the situation in these words

> Hier mussen wir allerdings eine zurzeit noch grosse Lucke in unseren Kenntnissen besonders hervorheben Wir wissen noch sehr wenig uber die Fähigkeiten der tierischen Zellen Aminosauren zu bilden Nur fur Glykokoll ist bewiesen, dass es aufgebaut werden kann Ferner wissen wir von Tryptophan, dass es offenbar von den Korperzellen nicht gebildet wird Ebenso scheinen die aromatischen Bausteine nicht ersetzbar zu sein Es muss somit der Möglichkeit gerechnet werden, dass die Zelle imstande ist, manchen Baustein zu erganzen und damit das zu Gebote stehende Aminosauregemisch besser verwertbar zu machen Durch die Hervorhebung dieser Lucken in unseren Kenntnissen uber den intermediären Zellstoffwechsel wird das Hypothetische in unseren Vorstellungen uber den Ablauf des Eiweissstoffwechsels im tierischen Organismus mit Absicht besonders betont[24]

The experimental records presented in the preceding pages suggest that we have in the past greatly underestimated the possibility of a transmutation or synthesis of amino-acids in the organism, and that these chemical processes may play a more significant part in nutrition than has been credited to them hitherto The realization of the possibility of a transmutation of amino-acids has an obvious bearing upon the question of the quantity of protein in the diet, for the arguments in favor of a liberal protein

[22] Cf Mendel *Ergeb d Physiol*, xi p 418, 1911, Cathcart *The Physiology of Protein Metabolism*, 1912

[23] McCollum *Amer Journ of Physiol*, xxix, p 215, 1911

[24] Abderhalden *Synthese der Zellbausteine in Pflanze und Tier*, Berlin, 1912

intake have in part been based upon the belief that all of the Bausteine must be supplied in adequate amounts The ability to be maintained over long periods on the chemically unique protein gliadin is an excellent illustration of the point raised

To what extent the daily "wear and tear" of metabolism may require a new supply of Bausteine to replace depleted tissue, remains a matter of conjecture at present Studies to determine the amount of nitrogenous material involved in what Rubner has termed the "Abnutzungsquote" show that it is not large in quantity The experiments here recorded give no answer to the possible extent of the synthesis of new amino-acids except perhaps in the case of glycocoll and lysine Further investigations with the various proteins must be undertaken to determine the *relative minimum quantities* of each of them essential for long continued maintenance Our foods ordinarily contained 18 per cent of protein—a proportion of the total intake found in the case of casein to be larger than the necessary minimum of about 6–7 per cent of the food Similar experiments are now in progress with gliadin and edestin

That a protein as unlike the tissue proteins as is gliadin can, in fact, serve for the construction of new tissues through the intervention of the metabolic processes of the mature animal is no longer to be doubted in view of an experiment reported in another paper,[25] in which a pair of rats maintained 178 days on gliadin as the sole protein in the diet produced four healthy young and successfully reared them This involved not only the construction of the tissues of the young, but also the production of the milk by which they were successfully nourished

The recent observation of Grafe and Schlapfer on the protein-sparing action of organic ammonium salts,[26] as well as those of Abderhalden,[27] taken in connection with the striking experiments of Knoop and Embden on the synthesis of individual amino-acids under purely experimental conditions, brings the synthetic powers of the animal organism into new prominence Indeed as Grafe remarks

[25] Osborne and Mendel This *Journal*, xii, p 473, 1912

[26] Grafe and Schläpfer *Zeitschr f physiol Chem*, lxxvii, p 1, 1912, Grafe *ibid*, lxxviii, p 485, 1912

[27] Abderhalden *ibid*, lxxviii, p 1, 1912

Da wir hier erst am Anfange unserer Kenntnisse auf einem ganz neuen Gebiete des intermediären Stoffwechsels und der synthetischen Leistungsfähigkeit des tierischen Organismus stehen, haben zunächst natürlich alle Deutungsversuche nur sehr untergeordnete Bedeutung und können nur als Arbeitshypothesen dienlich sein.

Further evidence of the physiological efficiency of animals maintained on single proteins is brought out by the fact that the capacity of reproduction has not been impaired in these animals and no obvious physical defects or unusual behavior were discernible.

The long records from the continued use of unchanged rations afford further confirmation of what we have earlier pointed out, namely, that monotony of diet is not necessarily a troublesome factor and is not of such importance in nutrition problems as is usually supposed.[28]

To what extent alimentary bacteria may intervene to furnish adequate building stones to the organism by utilizing the products of digestion found in the alimentary tract as their own nutritive pabulum and reconverting it by means of their eminent synthetic capacity into products suitable for the higher organisms cannot be definitely stated. It is not impossible that in the case of such substances as ammonium salts they may render some effective service by converting the nitrogen into other assimilable forms. We do not at present regard these bacterial possibilities as an adequate explanation of the nutritive success recorded, otherwise there is no apparent reason why a protein defective for growth should not be rendered efficient through this means. The contributions of the microorganisms to nutrient efficiency need further investigation and the possibilities have lately been clearly summarized by Armsby.[29]

We believe that experiments such as those reported in this paper show the possibility of approaching certain of the problems of nutrition by new and hitherto discredited methods of study. The exceptionally long periods of feeding and observation involved in our successful trials are in striking contrast with most of the previously published records. If we had been content to discon-

[28] Cf. Hart, McCollum, Steenbock and Humphrey: University of Wisconsin, Agricultural Experiment Station, Research Bulletin No. 17, 1911.

[29] Armsby: The Nutritive Value of the Non-protein of Feeding Stuffs, Bureau of Animal Industry, Bulletin 139, 1911.

tinue the experiments after a reasonable period many of the declines evidently associated with imperfections in the dietary, and readily checked by a change in feeding, would have escaped attention Such facts deserve to be considered in relation to current work in which the criteria of adequate nutrition on unusual diets such as amino-acid mixtures, etc , are sought in the nitrogen balance of the animals None of the records, so far as we are aware, extend over periods even half as long as some of ours which ultimately ended in nutritive failure Nitrogen balances may at times prove singularly deceptive, and give apparently favorable indications of equilibrium although the real nutritive status of the animal may be less promising This is brought out in the consideration of some of the animals reported in an earlier publication[30]

Now that an animal can be maintained for long periods on a single protein there is prospect of a successful consideration of those other factors in nutrition which are even more illusive The relative significance of the inorganic ions, and the qualitative value of the fats and carbohydrates which supplement the diet ought to be rendered amenable to study[31] In the case of the carbohydrates we have already made observations which suggest that it is no longer justifiable to consider these foodstuffs from a single standpoint without reference to their structural individuality The consideration of such facts must, however, be reserved for other communications

[30] Osborne and Mendel Carnegie Institution of Washington, Publication 156, Part I, 1911

[31] Cf Osborne and Mendel This *Journal*, xii, p 81, 1912

APPENDIX

Explanation of the Charts

The abscissae of the curves represent days and the ordinates, actual body weight (solid line) or food-intake (dotted line) in grams In some of the charts the average (normal) curve of growth, plotted from body weight data available for normally growing animals of the same sex, is represented by a broken line for comparison The food intake curve is plotted from the quantities of food eaten per week The numbers on the body weight curves indicate the time at which changes in the character of the feeding were instituted

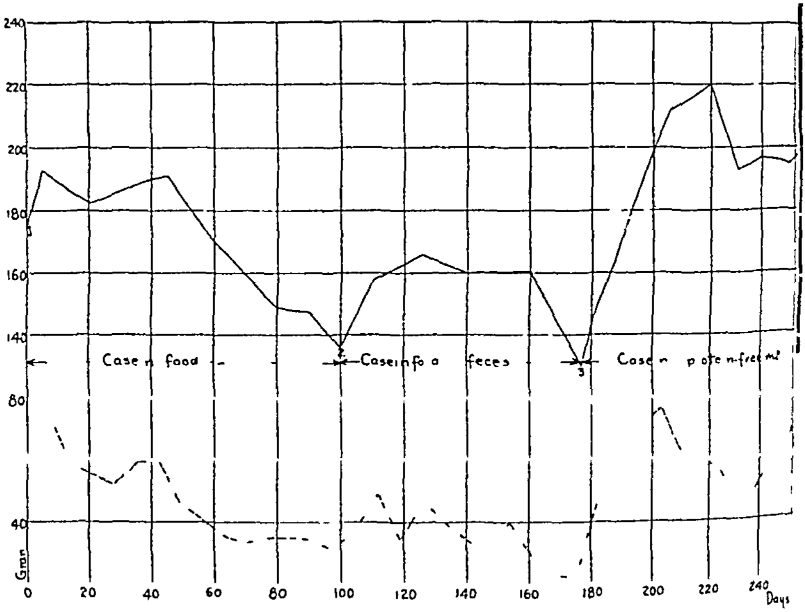


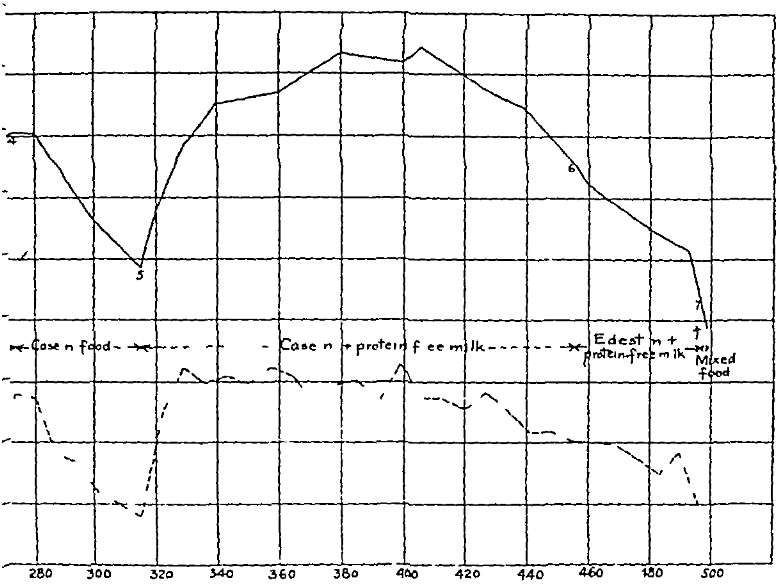
CHART 1, RAT 127 ♂, shows long-continued maintenance on the diets indicate diet was given each week The possible effect of this is discussed in Publicatio minated by diseased lungs after 499 days of experimental feeding

PERIODS 1 2 AND 4

	per cent
Casein (cow s milk)	18 0
Starch	32 5
Sucrose	21 9
Salt mixture I	2 6
Lard	25 0
	100 0

PERIODS

Casein (cow s milk)
Protein free milk
Starch
Agar
Lard



elow During period 2 about 1 gram of air-dry feces from rats on a mixed
6, p 61, Carnegie Institution of Washington The animal's life was ter-

D 5	PERIOD 6	
per cent		*per cent*
18 0	Edestin (hempseed)	18 0
28 2	Protein free milk	28 0
23 8	Starch	26 0
5 0	Lard	28 0
25 0		
100 0		100 0

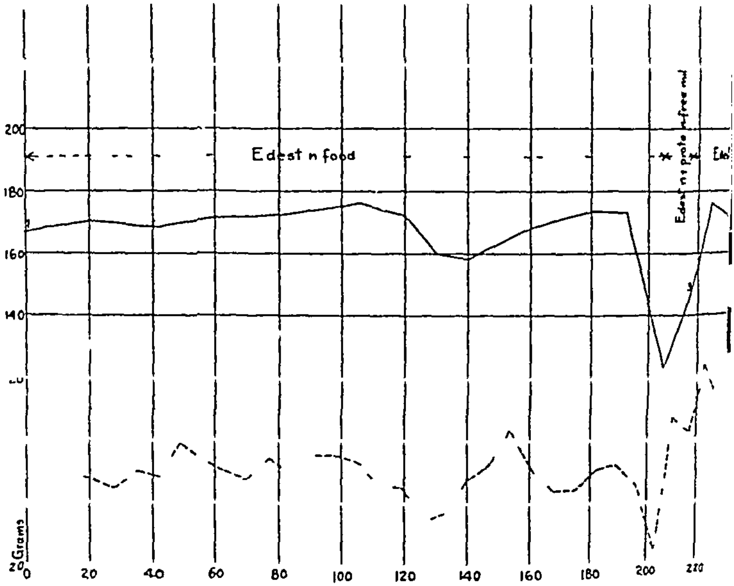


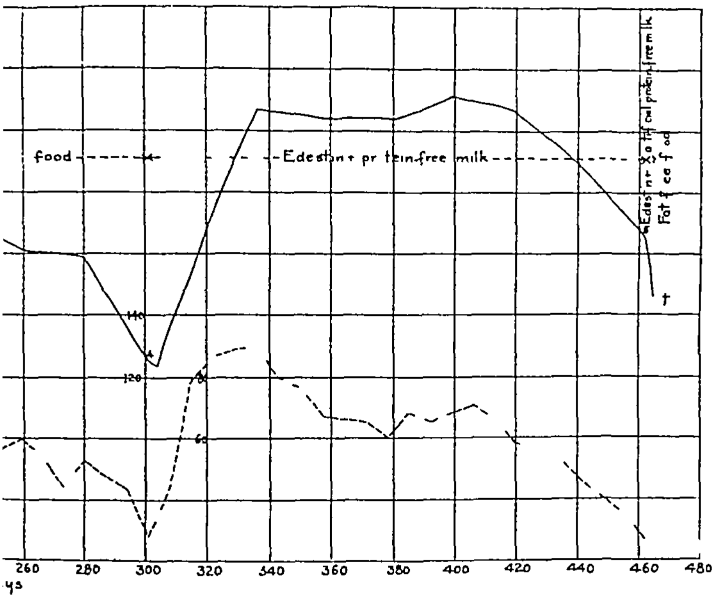
CHART 2, RAT 133 ♀, shows maintenance during 465 days on a diet in time the animal's life was terminated by a tumor of the spleen Note ganic salts and a part of the carbohydrate of the original food mixture

The diet during the different periods is shown below

PERIODS 1 AND 3

	per cent
Edestin (hemp seed)	18 0
Starch	29 5
Sucrose	17 0
Agar	5 0
Salt mixture I	2 5
Lard	28 0
	100 0

Edestin (hempseed)
Protein free milk
Starch
Agar
Lard



edestin from hempseed formed the sole protein At the end of that
d recovery in periods 2 and 4 when protein-free milk replaced the inor-

PERIOD 5

per cent		per cent
18 0	Edestin (hempseed)	22 0
28 2	Artificial protein free milk	29 5
20 8	Starch	28 5
5 0	Sucrose	20 0
28 0		
100 0		100 0

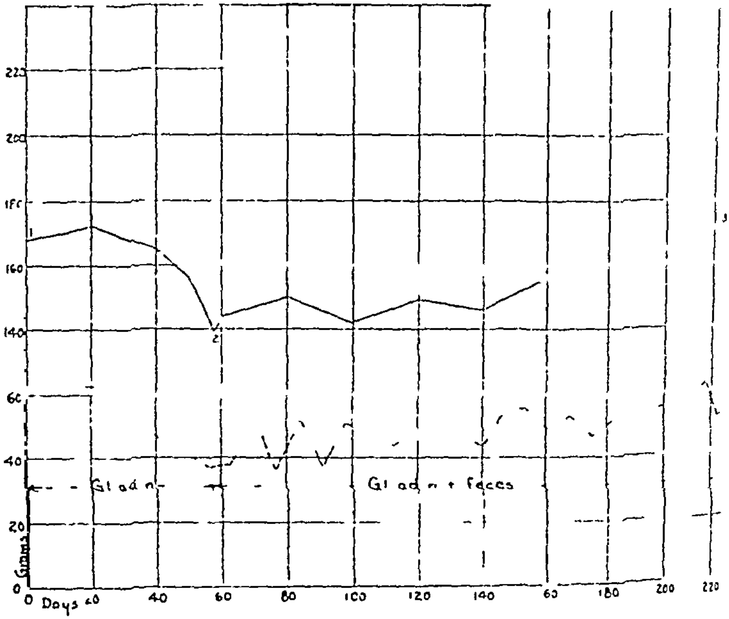
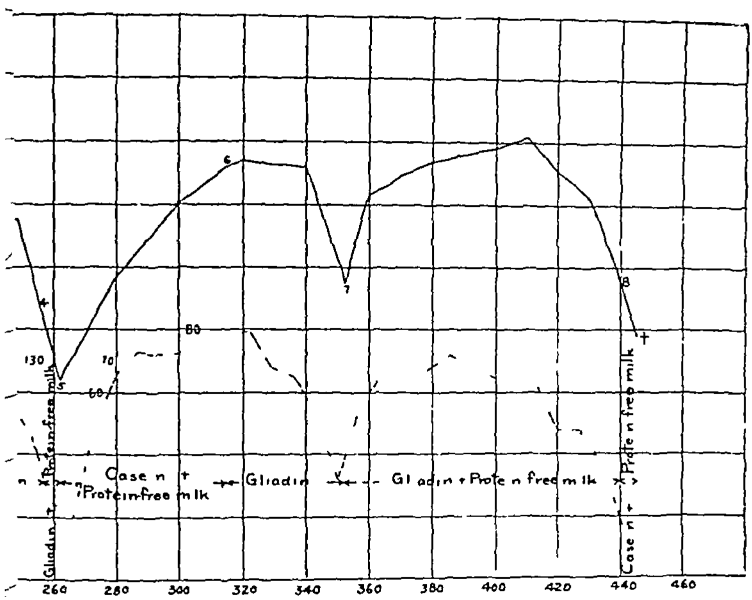


Chart 3, Rat 147 ♀, shows long continued maintenance on a diet cont
lungs after 445 days of experimental feeding

The diet during the different periods is shown below During period
on Chart 1

PERIODS 1 2 3 AND 6

	per cent	
Gliadin (wheat)	18 0	Gliadin (wheat)
Starch	29 5	Protein free milk
Sucrose	15 0	Starch
Agar	5 0	Agar
Salt mixture I	2 5	Lard
Lard	30 0	
	100 0	



gliadin as the sole protein The animal's life was terminated by diseased

all quantity of feces from rats on a mixed diet was supplied See legend

ND 7	per cent	PERIODS 5 AND 8	per cent
	18 0	Casein (cow s milk)	18 0
	28 2	Protein free milk	28 2
	20 8	Starch	23 8
	5 0	Agar	5 0
	28 0	Lard	25 0
	100 0		100 0

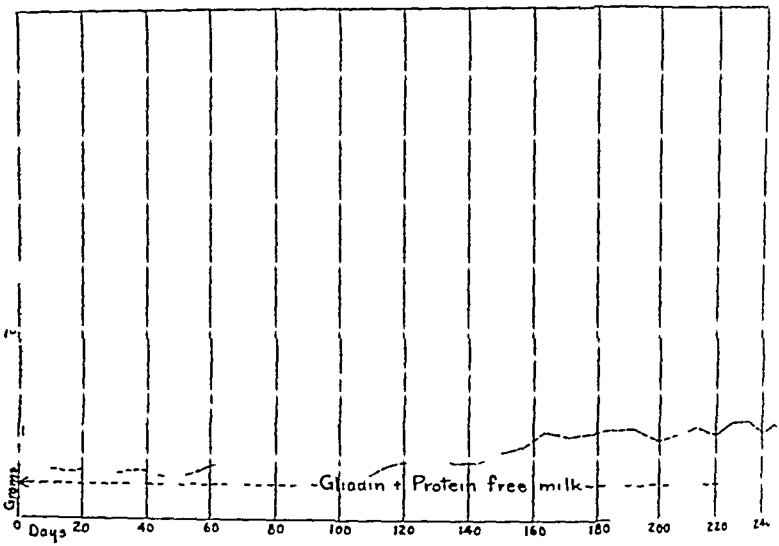
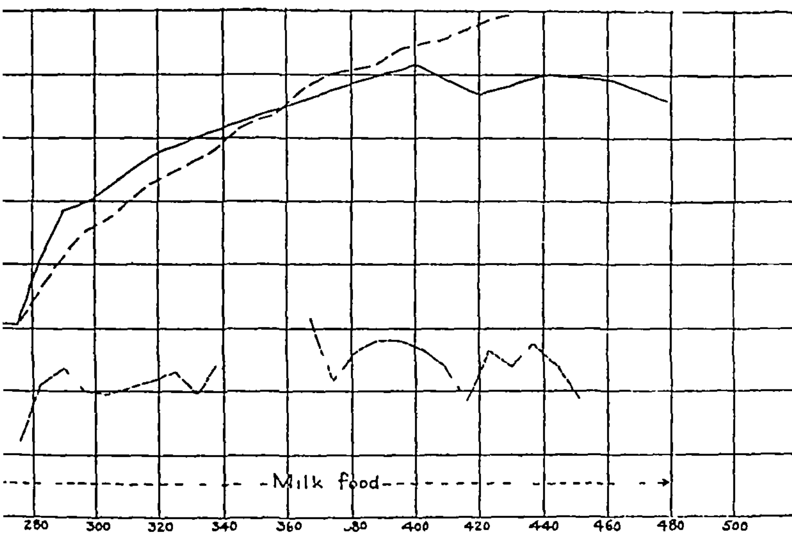


CHART 4, RAT 240 ♀, shows failure to make more than slight growth on normal rate after 276 days of stunting At this time the rat was 314 days o
The diet during periods 1 and 2 was

PERIOD 1

Gliadin (wheat)
Protein free milk
Starch
Agar
Lard



- containing gliadin as the sole protein, and capacity to resume growth at a
r age at which rats normally grow very little more

PERIOD 2

	per cent
ilk powder	60 0
tarch	16 0
ard	24 0
	100 0

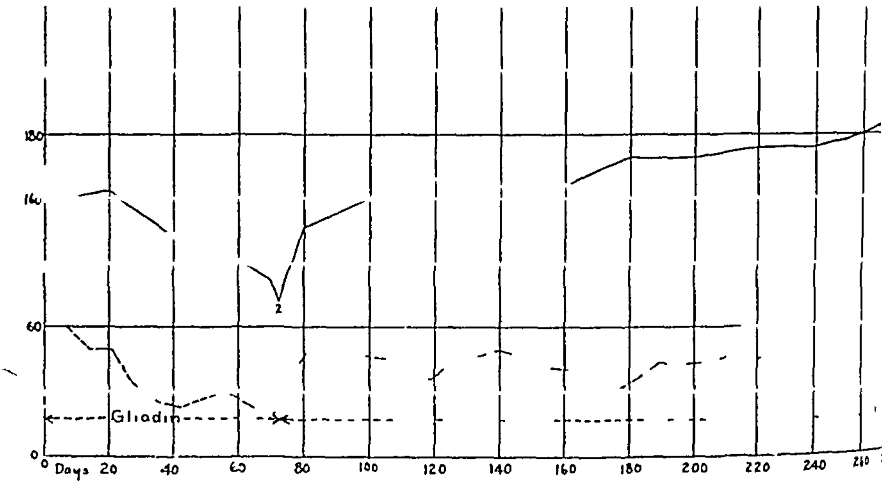
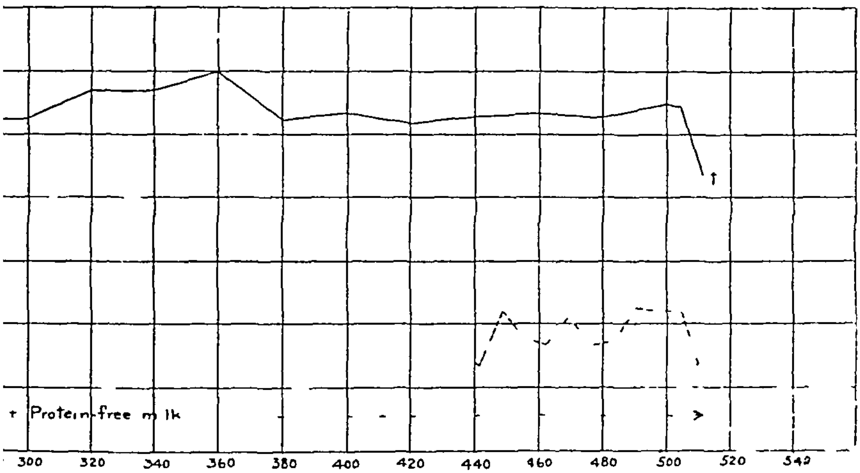


CHART 5, RAT 134 ♀, shows long continued maintenance on a diet containing
mental feeding by an ulcer of the pylorus

The diet during periods 1 and 2 was

PERIOD 1

Gliadin (wheat)
Starch
Sucrose
Agar
Salt mixture I
Lard



ι as the sole proteın The anımal's lıfe was te mınated after 511 days of experı

PERIOD 2

	per cent
adin (wheat)	18 0
ɔtein free milk	28 2
rch	20 8
ır	5 0
d	28 0
	100 0

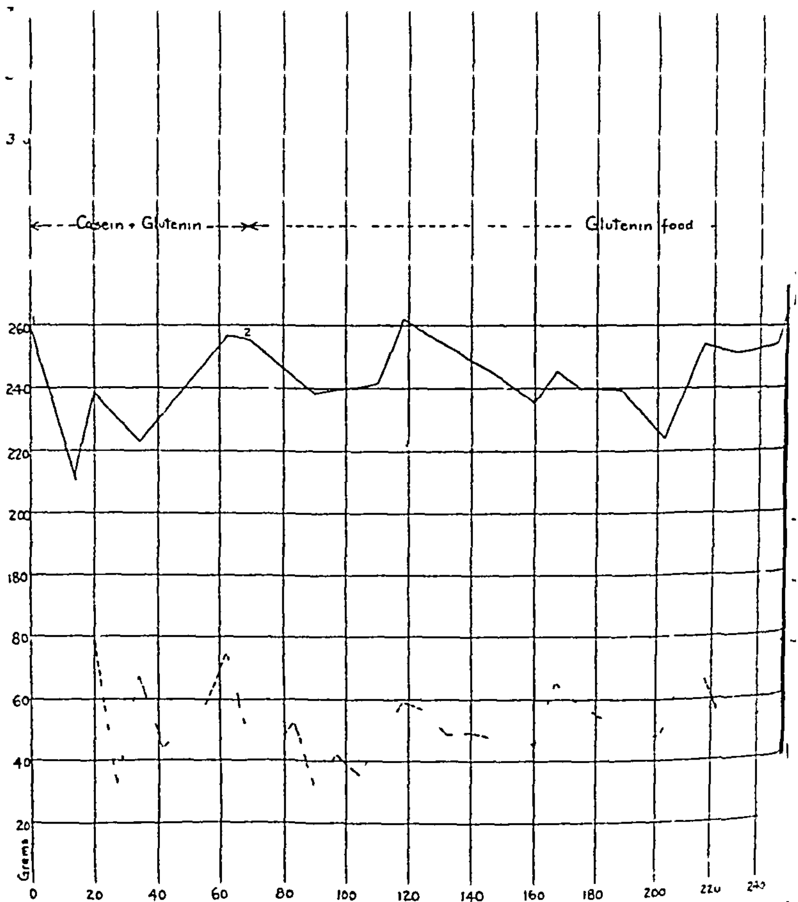
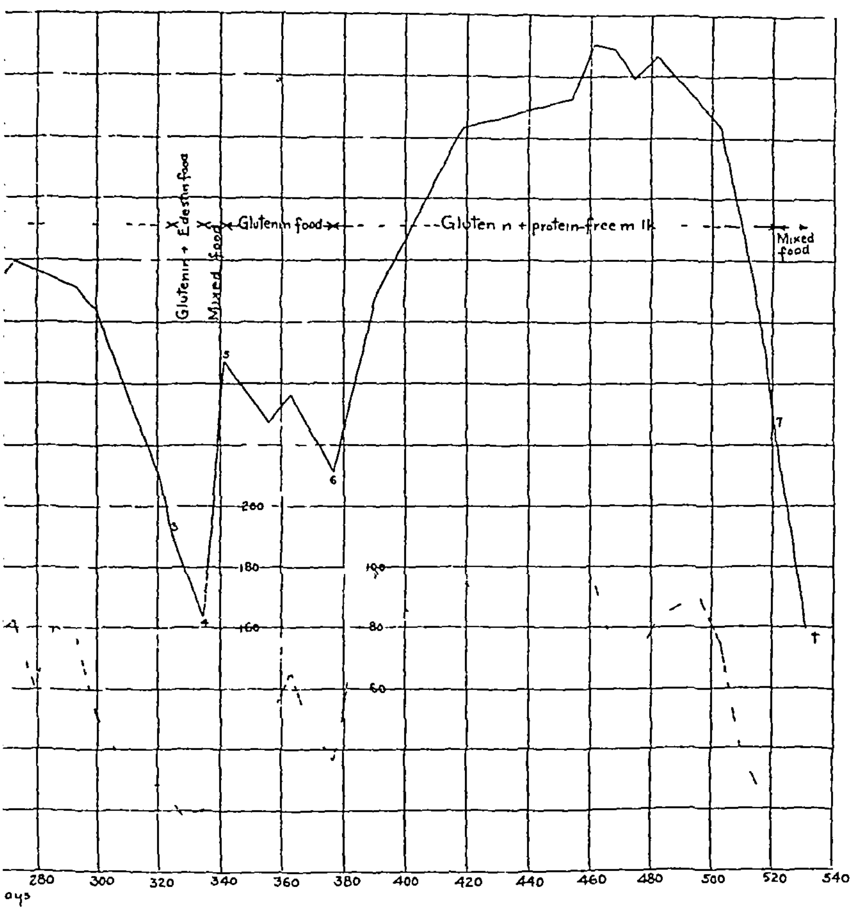


CHART 6, RAT 71 ♂, shows long-continued feeding of isolated foodstuffs and mal's life was terminated after 531 days of experimental feeding by an abscess or th
The diet during the different periods was as follows

PERIOD 1	*per cent*
Glutenin (wheat)	6 0
Casein (cow s milk)	12 0
Starch	24 5
Sucrose	15 0
Agar	5 0
Salt mixture I	2 5
Lard	35 0
	100 0

PERIODS 2 AND 5	*per cent*
Glutenin (wheat)	15 0
Starch	11 5
Sucrose	35 0
Agar	5 0
Salt mixture I	2 5
Lard	[illegible] 0
	[illegible]



long-continued maintenance on glutenin from wheat as the only protein The ani-
v which made eating impossible

PERIOD 3

	per cent
Glutenin (wheat)	9 0
Edestin (hemp seed)	9 0
Starch	33 5
Sucrose	18 5
Agar	5 0
Salt mixture I	2 5
Lard	22 5
	100 0

PERIODS 4 AND 7

Mixed

PERIOD 6

	per cent
Glutenin (wheat)	18 0
Protein free milk	28 2
Starch	23 8
Agar	5 0
Lard	25 0
	100 0

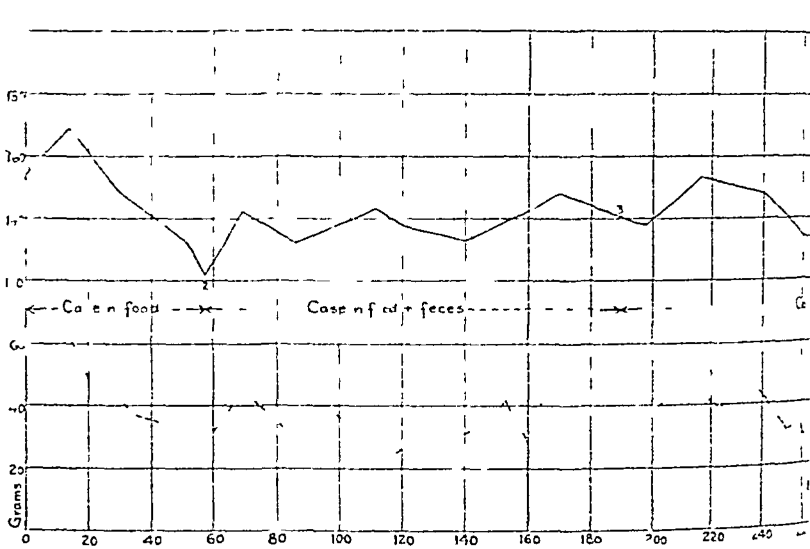
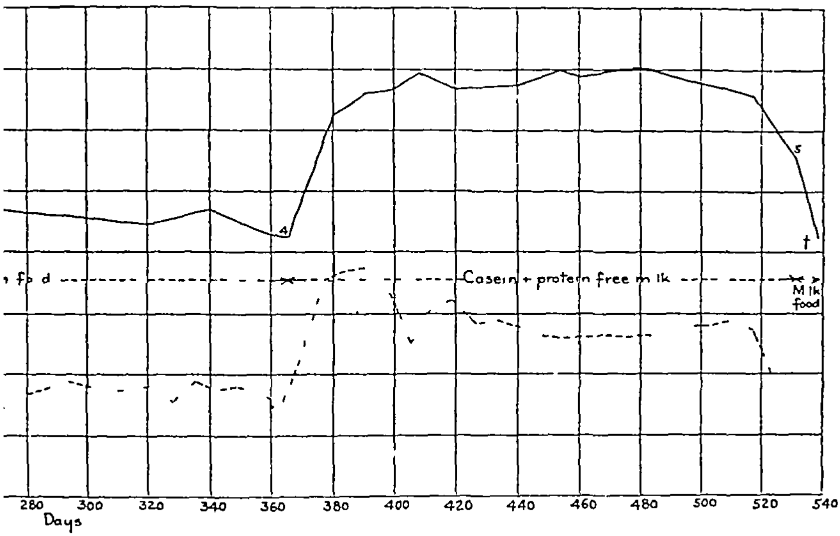


CHART 7, RAT 150 ♀, shows long-continued maintenance on a diet containing c
days of experimental feeding
The diet during the different periods is shown below During period 2 a small q
m

PERIODS 1 2 AND 3

	per cent
Casein (cow s milk)	18 0
Starch	32 5
Sucrose	21 9
Salt mixture I	2 6
Lard	25 0
	100 0

Casein (cow milk)
Protein free milk
Starch
Lard



in as its sole protein The animal's life was terminated by diseased lungs after 538

ntity of feces from rats on a mixed diet was supplied See legend on Chart 1

D 4

	per cent
	18 0
	28 0
	27 0
	27 0
	100 0

PERIOD 5

	per cent
Milk powder	60 0
Starch	12 0
Lard	28 0
	100 0

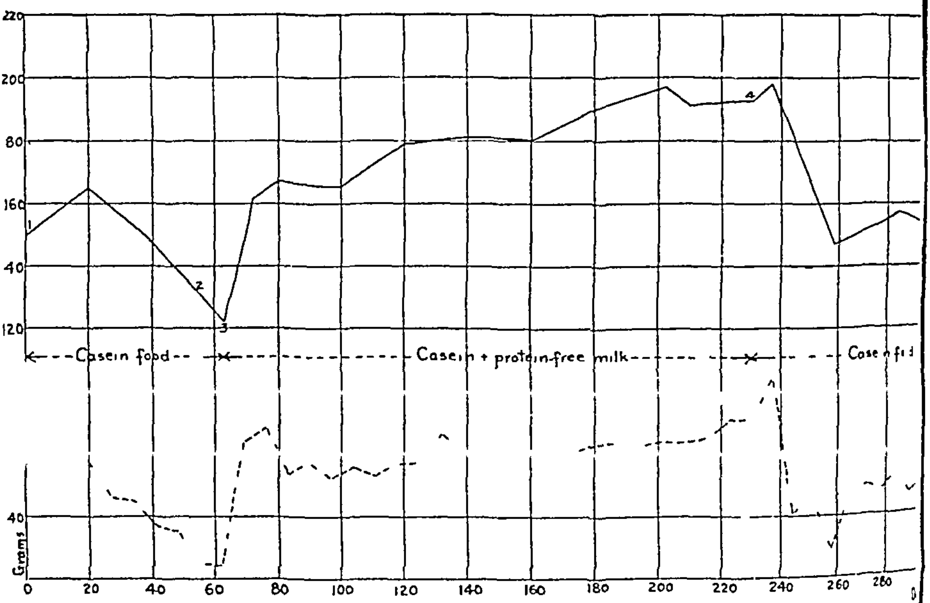
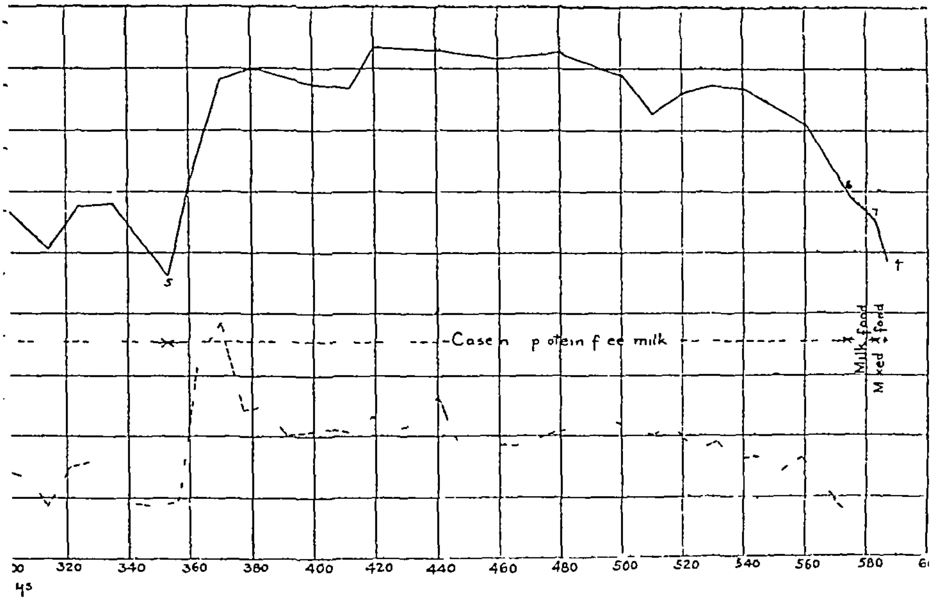


CHART 8, RAT 141 ♀, shows long-continued maintenance on a diet containing casein as it
seased lungs

The diet during the different periods is shown below

PERIODS 1 AND 4	per cent
Casein (cow s milk)	18 0
Starch	32 5
Sucrose	21 9
Salt mixture I	2 6
Lard	25 0
	100 0

PERIOD 2	per cent
Casein (cow s milk)	36 0
Starch	22 5
Sucrose	13 9
Salt mixture I	2 6
Lard	25 0
	100 0



sole protein After 587 days of experimental feeding the animal's life was terminated

PERIODS 3 AND 5

	per cent
Casein (cow's milk)	18 0
Protein free milk	28 0
Starch	27 0
Lard	27 0
	100 0

PERIOD 6

	per cent
Milk powder	60 0
Starch	12 0
Lard	28 0
	100 0

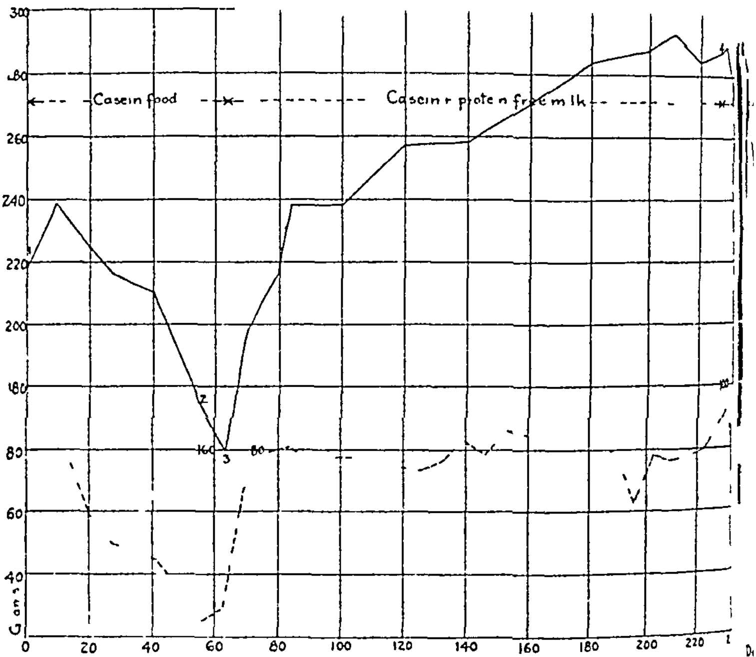
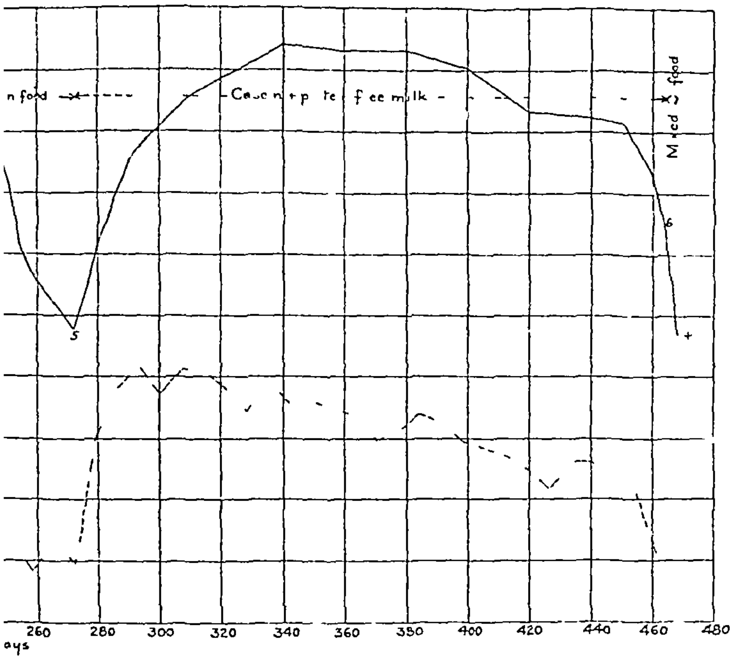


CHART 9, RAT 139 ♂, shows long-continued maintenance on a diet contai
mental feeding, but no cause for death was shown by the autopsy
The diet during the different periods is given below

PERIODS 1 AND 4

	per cent	
Casein (cow s milk)	18 0	Casein (cow s milk)
Starch	32 5	Starch
Sucrose	21 9	Sucrose
Salt mixture I	2 6	Salt mixture I
Lard	25 0	Lard
	100 0	



ig casein as its so e protein The animal died after 468 days of experi-

PERIODS 3 AND 5

per cent		per cent
36 0	Casein (cow s milk)	18 0
22 5	Protein-free milk	28 0
13 9	Starch	27 0
2 6	Lard	27 0
25 0		
100 0		100 0

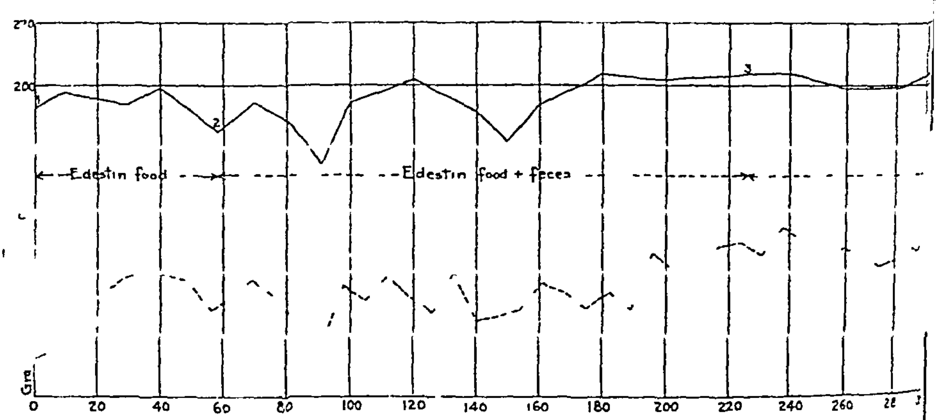
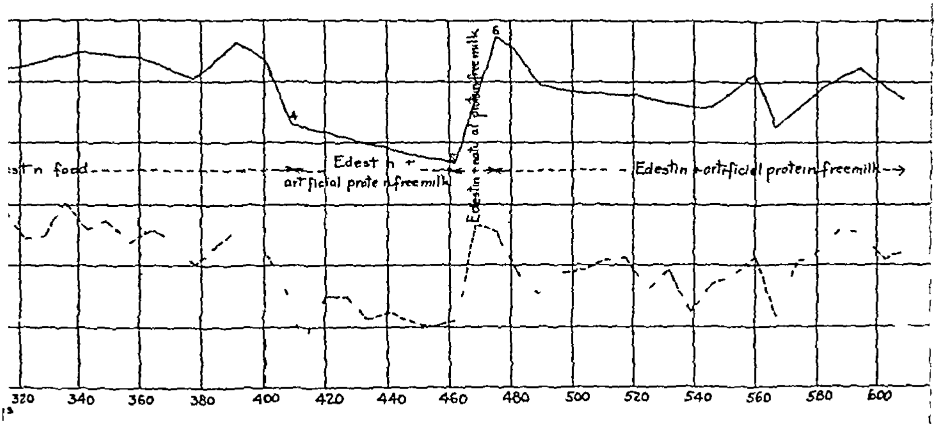


CHART 10, RAT 124 ♀, shows long-continued maintenance on a diet containing edestin as r
609 days of experimental feeding (which is, so far as we are aware, the longest record of
original weight During period 2 the rat received small quantities of feces from normally

PERIODS 1 2 AND 3

	per cent	
Edestin (hemp seed)	18 0	Edestin (hempseed)
Starch	29 5	Artificial protein-free
Sucrose	17 0	Starch
Agar	5 0	Lard
Salt mixture I	2 5	
Lard	28 0	
	100 0	



protein Note that this rat has had natural protein-free milk for only 14 days out of the enti
ind yet obtained) and also that at the end of this period the animal is somewhat above i
s See legend on Chart 1 This rat is still at its original weight after 637 days

AND 6		PERIOD 5
per cent		per cent
18 0	Edestin (hempseed)	18 0
29 5	Natural protein free milk	28 0
24 5	Starch	26 0
28 0	Lard	28 0
100 0		100 0

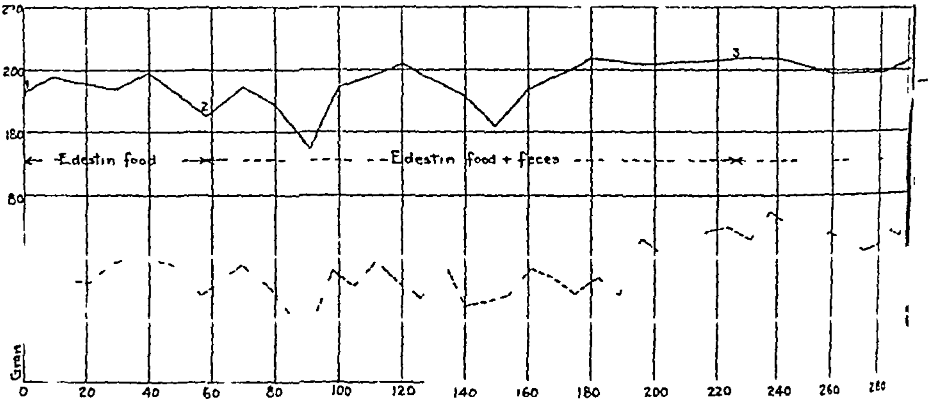
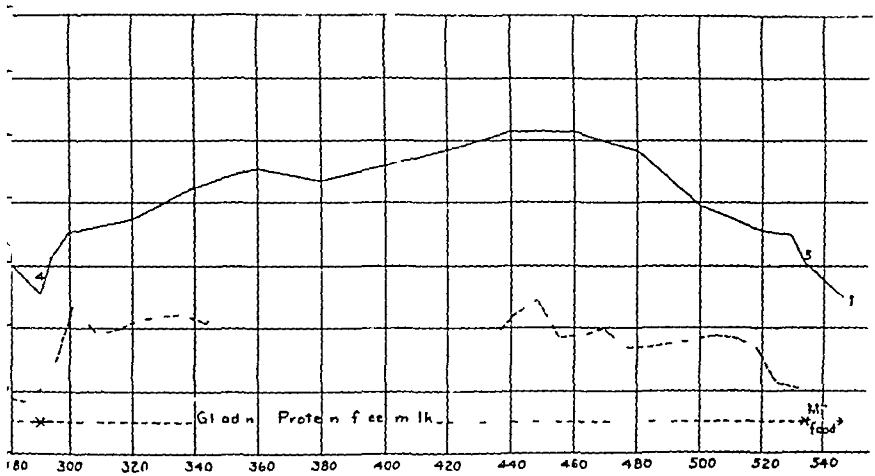


CHART 10, RAT 124 ♀, shows long-continued maintenance on a diet containing edestin as r
609 days of experimental feeding (which is, so far as we are aware, the longest record of
original weight During period 2 the rat received small quantities of feces from normally f

PERIODS 1 2 AND 3

	per cent	
Edestin (hemp seed)	18 0	Edestin (hempseed)
Starch	29 5	Artificial protein free m
Sucrose	17 0	Starch
Agar	5 0	Lard
Salt mixture I	2 5	
Lard	28 0	
	100 0	



adin as the sole protein The animal's life was terminated after 546 days of experi-
ne liver
ived small quantities of feces from normally fed rats See legend for Chart 1

4		PERIOD 5	
per cent			*per cent*
18 0	Milk powder		60 0
28 2	Starch		12 0
20 8	Lard		28 0
5 0			
28 0			100 0
100 0			

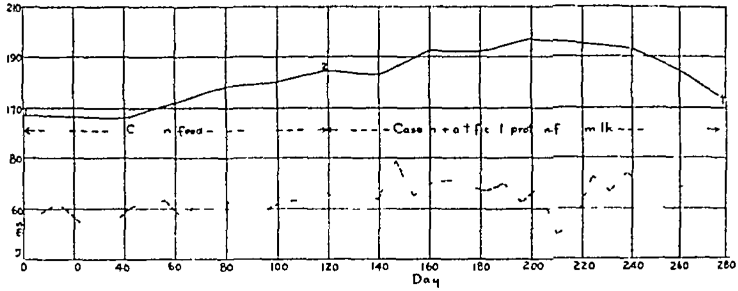


CHART 12 RAT 271 ♀, shows long continued maintenance on a purely artificial diet containing casein as its sole protein The animal died, after 277 days of experimental feeding with diseased kidneys

The diet during the different periods is shown below

PERIOD 1

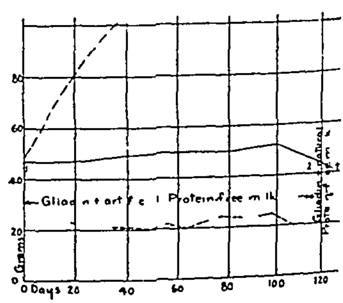
	per cent
Casein (cow s milk)	18 0
Starch	?4 5
Lactose	?4 0
Agar	5 0
Salt mixture I	. 5
Lard	26 0
	100 0

PERIOD 2

	per cent
Casein (cow s milk)	18 0
Artificial protein free milk	?6
Starch	26 4
Lard	26 0
	100 0

CHART 13 RAT 588 ♀ shows maintenance on a diet containing gliadin as the sole protein and an artificial imitation of the natural protein free milk After 114 days the artificial protein free milk was replaced by the natural, but the decline in weight which had begun was not stopped by this change The autopsy showed no adequate cause for death

The diet in periods 1 and 2 was



PERIOD 1

	per cent
Gliadin	18 0
Artificial protein free milk	30 0
Starch	?2 0
Lard	30 0
	100 0

PERIOD 2

	per cent
Gliadin	18 0
Protein free milk	8 0
Starch	26 0
La d	8 0
	100 0

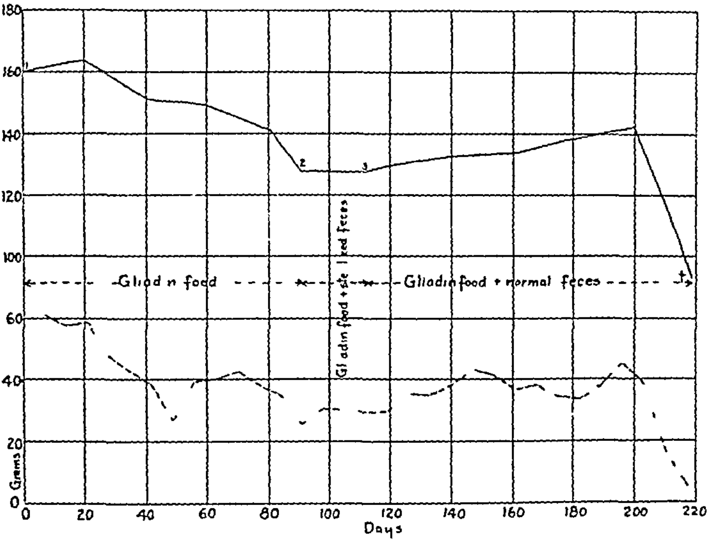


CHART 14, RAT 142 ♀, shows maintenance on a diet containing gliadin as its sole protein During periods 2 and 3 the rat received small quantities of feces from normally fed rats For a discussion of the effect of the sterilized and normal feces see Publication 156, p 62, Carnegie Institution of Washington The animal died suddenly after 219 days of experimental feeding, but unfortunately no autopsy was made

The diet was as follows

PERIODS 1 2 AND 3

	per cent
Gliadin (wheat)	18 0
Starch	29 5
Sucrose	17 0
Agar	5 0
Salt mixture I	2 5
Lard	28 0
	100 0

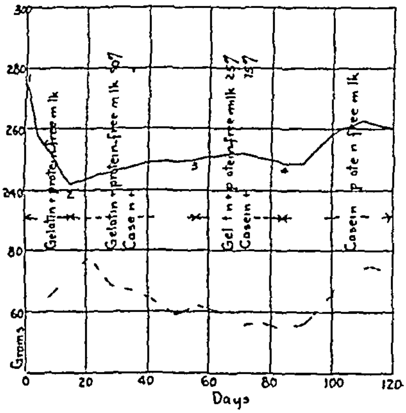


CHART 15, RAT 477 ♂, shows rapid decline on a diet containing gelatin as its sole protein, and subsequent maintenance and repair when the gelatin was either partially or entirely replaced by casein After 158 days of experimental feeding the animal's life was terminated by diseased lungs

The diet during the different periods is shown below

PERIOD 1

Gelatin food

	per cent
Gelatin (horn)	18 0
Protein free milk	28 0
Starch	27 0
Lard	27 0
	100 00

PERIOD 2

	per cent
Gelatin food (as in period 1)	50 0
Casein food (as in period 4)	50 0
	100 0

PERIOD 3

	per cent
Gelatin food (as in period 1)	25 0
Casein food (as in period 4)	75 0
	100 0

PERIOD 4

Casein food

	per cent
Casein (cow's milk)	18 0
Protein free milk	28 0
Starch	27 0
Lard	27 0
	100 0

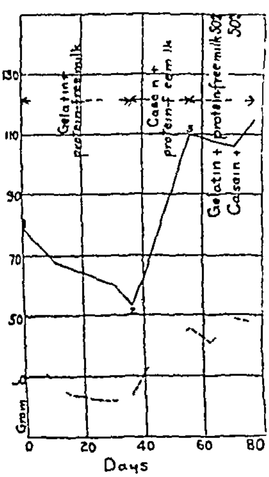


CHART 16, RAT 592 ♂, shows rapid decline on a diet containing gelatin as its sole protein, followed by normal growth when the gelatin is replaced by casein The animal's life was terminated after 119 days of experimental feeding by calculi in the bladder

The diet during the different periods is shown below

PERIOD 1

Gelatin food

	per cent
Gelatin (horn)	18 0
Protein free milk	28 0
Starch	27 0
Lard	27 0
	100 0

PERIOD 2

Casein food

	per cent
Casein (cow's milk)	18 0
Protein free milk	28 0
Starch	27 0
Lard	2[illegible] 0
	100 0

PERIOD 3

	per cent
Gelatin food (as in period 1)	50 0
Casein food (as in period 2	50 0
	100 0

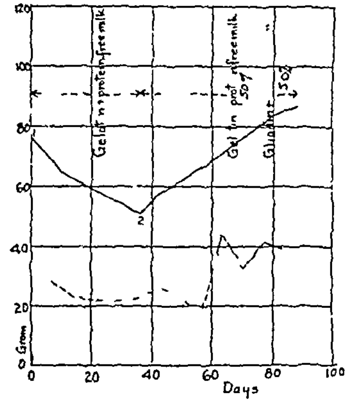


CHART 17, RAT 598 ♀, shows rapid decline on a diet containing gelatin as its sole protein, and recovery when one-half of the gelatin was replaced by gliadin, a protein incapable of inducing more than very slight growth when it forms the sole protein constituent of the dietary The animal's life was terminated by diseased kidneys after 120 days of experimental feeding

The diet during the different periods is shown below

PERIOD 1

	per cent
Gelatin (horn)	18 0
Protein free milk	28 0
Starch	27 0
Lard	27 0
	100 0

PERIOD 2

	per cent
Gelatin food (as in period 1)	50 0
Gliadin food	50 0
	100 0

Gliadin food

	gram
Gliadin (wheat)	18 0
Protein free milk	28 0
Starch	26 0
Lard	28 0
	100 0

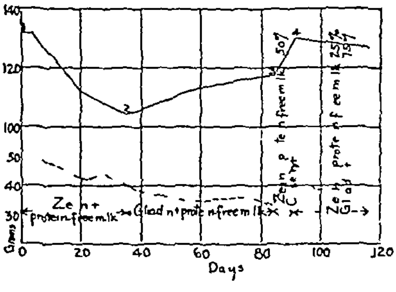


CHART 18, RAT 659 ♀, shows rapid decline on a diet containing zein as its sole protein, followed by recovery when the zein was entirely or partially replaced by gliadin or casein

The diet during the different periods is shown below

PERIOD 1

	grams
Zein (maize)	18 0
Protein free milk	28 0
Starch	24 0
Lard	30 0
	100 0
Water	15 cc

PERIOD 2

	per cent
Gliadin (wheat)	18 0
Protein free milk	28 0
Starch	24 0
Lard	30 0
	100 0

PERIOD 3

	per cent
Zein food (as in period 1)	50 0
Casein food	50 0
	100 0

PERIOD 4

	per cent
Zein food (as in period 1)	25 0
Gliadin food (as in period 2)	75 0
	100 0

Casein food

	grams
Casein	18 0
Protein free milk	28 0
Starch	29 0
Lard	25 0
	100 0

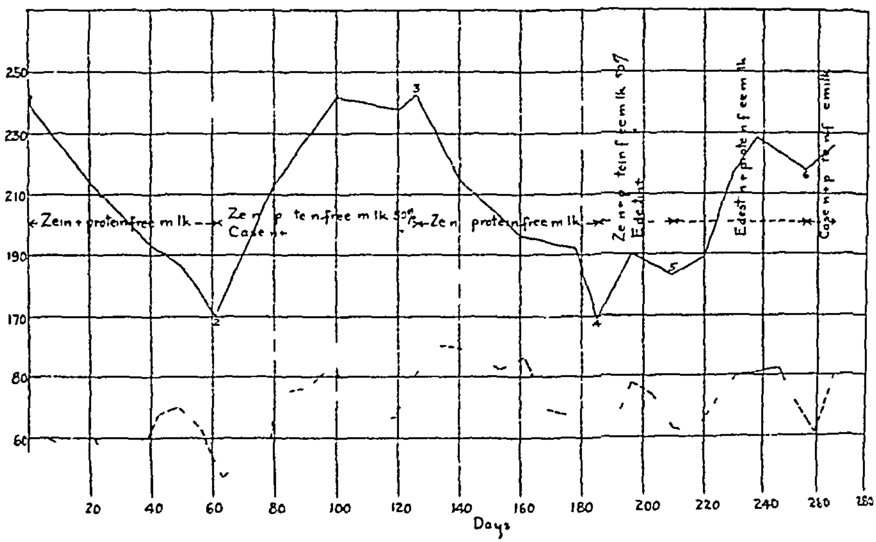


CHART 19, RAT 146 ♂ shows rapid decline on a diet containing zein as its sole protein, followed by speedy recovery when the zein was partially or entirely replaced by casein or edestin The experiment was terminated after 266 days of experimental feeding

The diet during the different periods is shown below

PERIODS 1 AND 3

	grams
Zein (maize)	18.0
Protein free milk	28.2
Starch	23.8
Agar	5.0
Lard	25.0
	100.0
Water	15 cc

PERIOD 2

	per cent
Zein food (as in period 1)	50.0
Casein food (as in period 6)	50.0
	100.0

PERIOD 4

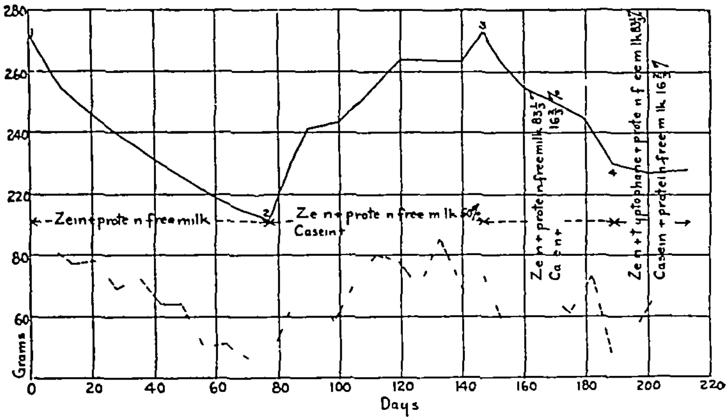
	per cent
Zein food (as in period 1)	50.0
Edestin food (as in period 5)	50.0
	100.0

PERIOD 5

	per cent
Edestin (hempseed)	18.0
Protein free milk	28.2
Starch	20.8
Agar	5.0
Lard	28.0
	100.0

PERIOD 6

	per cent
Casein (cow's milk)	18.0
Protein free milk	28.2
Starch	23.8
Agar	5.0
Lard	25.0
	100.0



Chart

CHART 20, RAT 475 ♂, shows rapid decline on a diet containing zein as its only protein, followed by recovery when one-half of the zein was replaced by casein Note that the decline in period 3 when only one-sixth of the zein was replaced by casein was immediately checked when 3 per cent of the zein was replaced by tryptophane

This experiment is still in progress

The diet during the different periods is shown below

PERIOD 1

	grams
Zein (maize)	18 0
Protein free milk	28 0
Starch	24 0
Lard	30 0
	100 0
Water	15 cc

PERIOD 2

	per cent
Zein food (as in period 1)	50 0
Casein food	50 0
	100 0

PERIOD 3

	per cent
Zein food (as in period 1)	83 33
Casein food (as in period 2)	16 67
	100 00

PERIOD 4

	per cent
Zein food (as below)	83 33
Casein food (as in period 2)	16 67
	100 00

Casein food

	grams
Casein (cow s milk)	18 0
Protein-free milk	28 0
Starch	27 0
Lard	27 0
	100 0

Zein food

	grams
Zein (maize)	17 46
Tryptophane	0 54
Protein free milk	28 00
Starch	24 00
Lard	30 00
	100 00
Water	15 cc

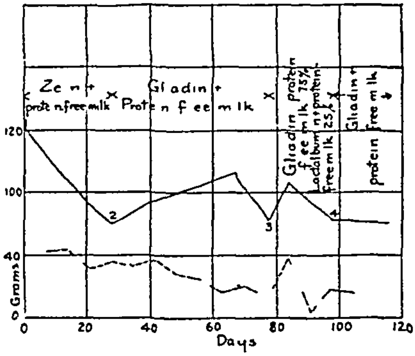


CHART 21, RAT 628 ♂, shows rapid decline on a diet containing zein as its sole protein, followed by maintenance when the zein was replaced by gliadin or lactalbumin

The diet during the different periods is shown below

PERIOD 1

	grams
Zein (maize)	18 0
Protein free milk	28 0
Starch	24 0
Lard	30 0
	100 0
Water	15 cc

PERIODS 2 AND 4

	per cent
Gliadin (wheat)	18 0
Protein free milk	28 0
Starch	26 0
Lard	28 0
	100 0

PERIOD 3

	per cent
Gliadin food (as in period 2)	75 0
Lactalbumin food	25 0
	100 0

LACTALBUMIN FOOD

	per cent
Lactalbumin (cow s milk)	18 0
Protein-free milk	28 0
Starch	29 0
Lard	25 0
	100 0

Following the directions of Siegfried, a substance was obtained from gelatin, resembling in every way kyrine sulphate, which was further purified by means of silver sulphate and barium hydrate The substance obtained after this purification had the following properties Its elementary composition resembled that of Siegfried's kyrine, its phosphotungstic compound crystallized in the same form as described by Siegfried for the same derivative of kyrine, on hydrolysis it gave arginine, lysine, glutaminic acid, glycocoll and proline The presence of the latter was demonstrated by the indirect method These amino-acids were combined in peptide linking to some extent The reason for this assumption is the following If the substance were a mixture of amino-acids the ratio of the amino nitrogen to the total nitrogen should have been 5 9 Since in arginine three nitrogen atoms do not function as primary amino nitrogen, and since the nitrogen of proline must be placed in the same class, in a mixture of the five named amino-acids containing nine nitrogen atoms, five possess the properties of primary amines in their behavior to nitrous acid

The ratio of amino nitrogen to total nitrogen in our substance was only from 32 to 33 per cent, indicating that some of the primary amino nitrogen atoms had ceased to function as such, presumably through the formation of peptide linkings

The next task was to ascertain whether or not the five amino-acids were combined in one or in more than one peptide If the five acids were linked into one peptide this should contain only two nitrogen atoms in the form of primary amino nitrogen, namely, that of the end-acid and the one in the ω-position in the lysine Thus a pentapeptide composed of the five named amino-acids requires a ratio of amino nitrogen to total nitrogen of 2 9 or of 22 per cent The substance obtained by us contained 32 per cent of amino nitrogen Hence it was composed of more than one peptide

These data, and the data obtained on the nitrogen partition after hydrolysis, led us to the belief that the substance consisted of two basic peptides one containing lysine and three mono-amino-acids, and the other arginine and one amino-acid This assumption was corroborated by the fact that it was possible to separate the mixture into two fractions by means of silver sul-

phate and barium hydrate One of these, arginine glutaminic acid peptide, was about 95 per cent pure The degree of purity was demonstrated by the ultimate analysis of its sulphate, by the ratio of amino nitrogen before and after hydrolysis, by the quantitative estimation of arginine contained in it and by the fact that both arginine and glutaminic acid were obtained from it on hydrolysis The second peptide has not yet been obtained in the same degree of purity The nitrogen partition of the pure peptide requires a ratio of 40 per cent of amino nitrogen, instead of which, the substance contained only about 37 per cent It is hoped that this peptide will also be obtained in a sufficient degree of purity before very long Work on the separation of the basic peptides from other proteins is at present in progress

The substance discussed here, with the $\frac{\text{Amino N}}{\text{Total N}}$ ratio of 32 per cent was not exactly the kyrine sulphate of Siegfried, but was chosen for the reason that it offered a more convenient material for presenting our arguments The kyrine prepared according to Siegfried by repeating the precipitation with alcohol five times had the ratio $\frac{\text{Amino N}}{\text{Total N}} = 23\ 6$ per cent By means of one treatment with silver sulphate and barium hydrate it was separated into two fractions, one of which consisted principally of the dipeptide, the other being a mixture having the ratio $\frac{\text{Amino N}}{\text{Total N}} = 32$ per cent *Hence it is evident that the substance obtained by us* according to the directions of Siegfried contained a higher proportion of the dipeptide than the mixture discussed in this communication

However, it should be born in mind that only slight changes in the conditions of hydrolysis may bring about considerable variations in the composition of the products obtained through it, and hence it is possible that the substance analyzed by Siegfried had a composition different from that of the substance obtained by us

The ratio of amino nitrogen to total nitrogen after hydrolysis

0 5 gram of the substance was hydrolyzed with 30 cc of a 20 per cent solution of hydrochloric acid for twenty hours over flame with return condenser The excess of hydrochloric acid was removed by distillation, the residue taken up in water, neutralized with sodium hydrate and brought to a volume of 50 cc

Total N 10 cc solution required 12 89 cc $\frac{N}{10}$ acid
N = 18 04 mgm
Amino N 10 cc solution gave 20 4 cc gas, 19°, 760 mm
N = 11 69 mgm

$\frac{\text{Amino N}}{\text{Total N}}$ 64 7 per cent

Nitrogen partition in the phosphotungstic acid precipitate and filtrate after hydrolysis

One gram of substance was hydrolyzed with a 20 per cent solution of hydrochloric acid for twenty hours over flame with return condenser After removing the hydrochloric acid by repeated concentration under diminished pressure the residue was taken up with 10 per cent sulphuric acid and a 10 per cent solution of phosphotungstic acid added The solution and precipitate so formed were then heated on the water bath until the precipitate was nearly all dissolved and then allowed to stand until the next day The nitrogen in the precipitate and filtrate was determined according to Kjeldahl

The precipitate required 44 60 cc of $\frac{N}{10}$ acid and the filtrate 25 92 cc of $\frac{N}{10}$ acid Hence the ratio of nitrogen in the phosphotungstic precipitate to the total nitrogen $= \frac{44\ 60}{70\ 52} = 63\ 25$ per cent

Amino nitrogen ratio in the phosphotungstic acid precipitate and filtrate

In a second portion the ratio of amino nitrogen to total nitrogen in the phosphotungstic acid precipitate and filtrate after acid hydrolysis was determined The precipitate was brought into solution with the aid of dilute alkali and brought to a volume of 30 cc

Total N 10 cc solution gave 9 19 cc $\frac{N}{10}$ acid N = 38 59 mgm *in toto*

Amino N 10 cc solution gave 12 cc gas, 20°, 760 mm N = 20 52 mgm *in toto*

$\frac{\text{Amino N}}{\text{Total N}}$ 53 1 per cent

The filtrate was concentrated to a volume of 50 cc

Total N 35 cc solution required 15 cc $\frac{N}{10}$ acid N = 30 0 mgm *in toto*

Amino N 10 cc solution gave 7 9 cc gas, 19°, 760 mm N = 22 53 mgm *in toto*

$\frac{\text{Amino N}}{\text{Total N}}$ 75 1 per cent

Hydrolysis of the kyrine fraction aiming to isolate the individual components

About 45 grams of the sulphate were hydrolyzed with a 20 per cent solution of hydrochloric acid for twenty-four hours The solution was then treated with phosphotungstic acid in the manner before described, the precipitate was washed chlorine-free with 5 per cent sulphuric acid, and the phosphotungstic and sulphuric acids were removed quantitatively with barium hydrate The resulting liquid was concentrated to a small volume and to a portion an alcoholic solution of picrolonic acid was added and allowed to stand over night The precipitate so obtained was recrystallized out of water and analyzed

Substance, 0 1600 gram, CO_2, 0 2541, H_2O, 0 0706

	Calculated for $C_6H_{14}N_4O_2$ $C_{10}H_8N_4O_5$	Found
C	43 82	43 23
H	5 05	4 90

Lysine was isolated from the phosphotungstic acid precipitate according to Kossel's method and obtained as the picrate It was identified by determining the amino nitrogen

Substance, 0 0930 gram, gas, 12 8 cc , 20°, 764 mm

	Calculated for $C_6H_{14}N_2O_2$ $C_6H_2(NO_2)_3OH$	Foun
Amino N	7 48	7

Glutaminic acid was separated from the phosphotungstic acid filtrate as hydrochloride after removal of the sulphuric and phosphotungstic acids quantitatively by barium hydrate

0 1500 gram substance gave 0 1153 gram silver chloride

	Calculated for $C_5H_9NO_4$ HCl	Found
Cl	19 31	19 01

The excess of hydrochloric acid was removed by concentration under diminished pressure and the remainder with silver sulphate The silver was removed with hydrogen sulphide and the sulphuric acid with barium hydrate and the volume brought to 200 cc

Total N 5 cc required 17 85 cc $\frac{N}{10}$ acid N = 24 94 mgm
Amino N 5 cc gave 32 2 cc gas, 20°, 760 mm N = 18 30 mgm

The ratio of amino nitrogen to total nitrogen in the phosphotungstic acid filtrate = 73 3 per cent or 26 7 per cent of the nitrogen is non-amino nitrogen This indicates the presence of proline or oxyproline

The copper salts were formed from the remainder of the filtrate and extracted with hot alcohol On standing a large portion crystallized out The portion soluble in hot and cold alcohol appeared on analysis to be largely the copper salt of proline or oxyproline It was dissolved in 20 cc of water

Total N 10 cc required 20 43 cc $\frac{N}{10}$ acid N = 28 60 mgm
Amino N 10 cc gave 17 cc gas, 18°, 764 mm N = 9 81 mgm

$\frac{\text{Amino N}}{\text{Total N}}$ = 34 3 or 65 7 per cent of the nitrogen of the phospho tungstic acid filtrate is non-amino

The nitrogen content of the crystals which separated from the hot alcohol did not correspond to any of the ordinary *amino-acids*

0 0964 gram substance required 7 3 cc $\frac{N}{10}$ acid N = 10 22 mgm
Total N 10 60 per cent
0 0991 gram substance gave 18 4 cc gas, 20°, 764 mm
N = 10 53 mgm
Amino N 10 63 per cent

The analysis of the copper salt of the portion insoluble in hot and cold alcohol indicated a mixture, probably of alanine and glycocoll

0 2154 gram substance gave 0 2146 gram CO_2, 0 0910 gram H_2O

	Calculated for $(C_7H_4NO_2)_2Cu$	Calculated for $(C_3H_6NO_2)_2Cu$	Found
C	25 00	30 00	27 28
H	4 16	5 00	4 60

The results of the hydrolysis show that arginine, lysine and glutaminic acid are certainly present and probably also proline Glycocoll may also be present and perhaps alanine

Preparation of the dipeptide

The precipitate obtained on treatment of the solution of the original kyrine with silver sulphate and barium hydrate contained the dipeptide It was freed from silver and barium in the manner described earlier in the paper and transformed into the sulphate in the same manner as the original kyrine The dipeptide nature of the substance was based on the $\frac{\text{Amino N}}{\text{Total N}}$ ratio before and after hydrolysis, on the fact that on hydrolysis it yielded arginine and glutaminic acid and on its elementary composition A dipeptide composed of arginine and glutaminic acid should contain only one free primary amino group out of five nitrogen atoms in the molecule, or the $\frac{\text{Amino N}}{\text{Total N}}$ is calculated at 20 per cent The values found on analysis of the substance only slightly exceeded that value

The ratio of the nitrogen in the form of amino-acids to the basic nitrogen is calculated also at 20 per cent, and the analyzed body showed the value of the nitrogen of amino-acids to represent 21 1 per cent of the total nitrogen

Furthermore, on hydrolysis of the dipeptide, free arginine and free glutaminic acid are formed, hence the mixture of the two contains twice the number of amino groups as compared with the original number The value found on hydrolysis of the analyzed body only slightly exceeded the theoretical value

Finally the arginine determination made directly on the peptide gave results deviating only slightly from the value theoretically calculated

Elementary analysis of the dipeptide

The dipeptide was analyzed as sulphate It was dried under diminished pressure at room temperature over sulphuric acid for several days and then at the temperature of boiling chloroform for two days and at that of boiling carbon tetrachloride for five days

Substance, 0 2407 gram, CO_2, 0 2943, H_2O, 0 1242
Substance, 0 1808 gram, CO_2, 0 2160, H_2O, 0 0916
0 3209 gram substance required 40 1 cc $\frac{N}{10}$ acid = 56 14 mgm N
0 4033 gram substance gave 0 2712 gram $BaSO_4$
0 1952 gram substance gave 0 1276 gram $BaSO_4$

	Calculated for $(C_{11}H_{21}N_5O_4)H_2SO_4$	Found
C	32 90	32 97
H	5 73	5 72
N	17 40	17 48
S	8 20	9 11

Hydrolysis of the dipeptide indicating the nitrogen partition before and after hydrolysis

Two solutions of the sulphate were prepared, the first containing approximately 0 5 gram of the sulphate in 25 cc and the second, 1 0 gram in 25 cc These solutions were used for the determination of the amino nitrogen before hydrolysis A third solution containing approximately 2 5 grams of the sulphate was hydrolyzed with hydrochloric acid in the usual manner and the nitrogen partition in the phosphotungstic acid precipitate and filtrate determined in the manner described for the "purified kyrine" fraction The ratio of amino nitrogen to total nitrogen was also determined in the same manner

Before hydrolysis

(*a*) Total N 10 cc solution required 17 cc $\frac{N}{10}$ acid N = 23 80 mgm
Amino N 10 cc solution gave 8 7 cc gas, 20°, 760 mm N = 4 96 mgm

$\frac{\text{Amino N}}{\text{Total N}}$ 20 8 per cent

(*b*) Total N 5 cc solution required 21 4 cc $\frac{N}{10}$ acid N = 29 96 mgm
Amino N 10 cc solution gave 22 5 cc gas, 30°, 758 mm N = 12 23 mgm

$\frac{\text{Amino N}}{\text{Total N}}$ 20 4 per cent

After hydrolysis

(1) *Nitrogen partition in phosphotungstic acid precipitate and filtrate* Total volume of the solution, 50 cc 5 cc solution required 21 78 cc $\frac{N}{10}$ acid Total N = 304 92 mgm

Total N in filtrate from phosphotungstic acid precipitate Total volume of solution, 100 cc 25 cc solution required 11 55 cc $\frac{N}{10}$ acid N = 16 17 mgm Total N = 64 68 mgm

The ratio of the nitrogen in the phosphotungstic acid filtrate to the total nitrogen = 21 1 or the ratio of the nitrogen in the phosphotungstic acid precipitate to the total nitrogen = 78 9 per cent

Allowing for the correction of the solubility of arginine phosphotungstate, one finds the nitrogen value of the phosphotungstic precipitate to constitute 79 7 per cent of the total nitrogen

(2) *The ratio of amino nitrogen to total nitrogen* This determination was made on a second portion hydrolyzed as previously described

Total N	10 cc solution required 21 90 cc $\frac{N}{10}$ acid	N = 30 66 mgm
Amino N	10 cc solution gave 24 cc gas, 20°, 760 mm	N = 13 68 mgm

$$\frac{\text{Amino N}}{\text{Total N}} \qquad 44\ 4 \text{ per cent}$$

Hydrolysis of the dipeptide

100 cc of an aqueous solution of the dipeptide containing 1 22 grams of nitrogen were concentrated to 20 cc and 2 grams of sulphuric acid added This was hydrolyzed by heating for five hours in a sealed tube Water was added so that the solution finally contained 5 per cent sulphuric acid and then was precipitated with phosphotungstic acid After standing for two days in the refrigerator, the precipitate was filtered off in the cold and washed with 5 per cent sulphuric acid The phosphotungstic acid was removed from the filtrate by shaking out with ether, the ether distilled off, and the sulphuric acid removed quantitatively with barium hydrate The resulting solution was concentrated under diminished pressure to a small volume and alcohol added, when a portion crystallized out This crystallized portion was dissolved in water and converted into the picrolonate and analyzed

Substance, 0 0998 gram, CO_2, 0 1604 gram, H O, 0 0338 gram

	Calculated for $C_5H_9NO_4$ $C_{10}H_8N_4O_5$	Found
C	43 75	4 38
H	4 16	?

The mother liquors were also converted into the picrolonate These analyzed for a mixture of an inorganic picrolonate and impure glutaminic acid

Ash-free substance, 0 1038 gram, CO_2, 0 1702 gram H_2O 0 0274 gram, Ash, 0 0022 gram

C	44 70 per cent
H	2 95 per cent
Ash	2 20 per cent

The phosphotungstic acid precipitate was suspended in water, decomposed with barium hydrate and the acid removed quantitatively The resulting solution was concentrated under diminished pressure and picrolonic acid added The resulting crystals were filtered off, washed, dried and analyzed

Substance, 0 1196 gram, CO_2, 0 1804 gram, H_2O, 0 0420 gram

	Calculated for $C_6H_{14}N_4O_2(C_{10}H_8N_4O_5)_2$	Found
C	44 40	45 05
H	4 27	4 30

Arginine content of the dipeptide

The arginine was determined directly on the peptide without preceding hydrolysis 10 cc of an aqueous solution of the substance were boiled in a Kjeldahl flask with 20 cc water and 15 grams of potassium hydrate in the manner described by Van Slyke[3] for arginine determination At the end of ten hours water was added to the flask and the distillation continued for another hour Of the original aqueous solution of the dipeptide 10 cc were used for the arginine determination and 5 cc for the total nitrogen

Total N 5 cc solution required 35 cc $\frac{N}{10}$ acid N = 49 mgm
Arginine N 10 cc solution boiled with 20 cc water and 15 grams KOH neutralized 26 75 cc $\frac{N}{10}$ acid = 37 45 mgm N Total arginine N = 74 90 mgm

	Calculated	Found
Total Arginine N	80 0	76 5 per cent

[3] This *Journal*, x, p 26, 1911

A CONTRIBUTION TO OUR KNOWLEDGE OF THE GAS METABOLISM OF BACTERIA

FIRST PAPER

THE GASEOUS PRODUCTS OF FERMENTATIONS OF DEXTROSE BY B COLI, BY B TYPHOSUS AND BY BACT WELCHII

By FREDERICK G KEYES AND LOUIS J GILLESPIE

(*From the Biological Laboratory of Brown University*)

(*Received for publication, October 9, 1912*)

Earlier investigations

B coli The literature on the gases produced by *B coli* on various culture media has already been reviewed by one of us [1] It was found that very little work had been attempted on this subject by investigators familiar with the properties of gases

Harden[2] found that, when *B coli* was grown anaerobically on a non-albuminous dextrose medium based on asparagine, the asparagine was reduced to ammonium succinate with a consequent lessened evolution of hydrogen He therefore made use of media made of beef broth and of Witte's peptone and concluded, from a study of the solid, liquid and gaseous products, that on these media dextrose was decomposed with the formation (if not evolution) of equal volumes of hydrogen and of carbon dioxide His gas analyses showed for the most part more hydrogen than carbon dioxide

One of us[3] determined for several strains of *B coli* the quantities and the composition of gas obtained in various incubation periods

[1] Keyes *Journ of Med Res*, xxi (N S xvi), p 69, 1909 In this communication the composition of the culture medium was unfortunately given incorrectly The medium always contained, in addition to the constituents noted, 1 per cent of Merck's "Highest Purity" dextrose

[2] Harden *Trans Jenner Inst*, 1899, ii, p 126, *Journ Chem Soc* (Transactions), lxxix, p 610, 1901

[3] Keyes *Journ of Med Res*, xxi (N S xvi), p 69, 1909

by an exact method from vacuum fermentations of dextrose A non-albuminous asparagine medium was used for the most part, but a dextrose meat bouillon was also tested, and it was found that a larger percentage of hydrogen was indeed evolved from the albuminous medium but that even on this medium carbon dioxide was produced in considerably greater volume than hydrogen [4]

B typhosus Hesse[5] studied the gas metabolism of this organism by growing it in the presence of air and drawing off samples of gas from day to day for analysis He stated that carbon dioxide is evolved and that oxygen is absorbed but he did not calculate his analytical results We calculate from his protocols that in one instance as much as 18 cc of carbon dioxide was evolved aerobically from 33 cc of "glycerin agar-agar"

Harden[6] detected no production of gas from anaërobic fermentations of dextrose by *B typhosus* and Pakes and Jollyman[7] detected formation of gas from sodium formate—the supposed intermediate product between dextrose and carbon dioxide and hydrogen

Bact welchii Dunham[8] analyzed the gas evolved by this organism from 1 per cent dextrose bouillon in Smith fermentation tubes The ratio CO_2 H_2 was 0 43, but his results are not comparable with ours because he disregarded the loss of carbon dioxide due to solution in the medium and to diffusion from the open end of the fermentation tube

Technique

Organisms Cultures were kept in a vigorous state of growth by means of frequent transfers and isolations by the plate method The medium used for *B typhosus* and *Bact welchii* was the ordinary meat extract peptone agar, in the case of *B coli* it consisted

[4] We shall give below an analysis from a fermentation on a Witte's peptone medium, which gave practically the same figures as the meat bouillon We have always found more CO_2 than H_2, and attribute Harden's contrary result, as also his negative result with *B typhosus*, to insufficient removal of dissolved carbon dioxide from the culture fluid

[5] Hesse *Zeitschr f Hyg*, xv, p 17, 1893

[6] Harden *Journ Chem Soc* (Transactions), lxxix, p 610, 1901

[7] Pakes and Jollyman *Journ Chem Soc* (Transactions), lxxix, p 459, 1901

[8] Dunham *Johns Hopkins Hospital Bull*, viii, p 68, 1897

of 1 per cent dextrose, 0 2 per cent disodium phosphate and 1 5 per cent agar, with either 1 per cent asparagine for experiments on asparagine media or 1 per cent ammonium lactate for experiments on lactate media, and was made neutral to litmus with sodium hydrate Test cultivations of *B coli* or of *B typhosus* were started from a loopful of culture taken from the surface of a twenty-four-hour growth on slant agar *Bact welchii* fermentations were started from stab cultures Subcultures from the test cultivations were never used for further tests The organisms answered the usual identification tests

Culture fluids The culture fluids were sterilized in the absence of air in fermentation bulbs (to be described) in streaming steam by the intermittent method The reaction after boiling was slightly acid to litmus, except in certain experiments (noted in the protocols) in which the reaction was made just neutral to phenolphthalein by the addition of normal sodium hydrate When alkali was added, the amount of carbon dioxide thus introduced was calculated from a determination of the actual volume of carbon dioxide liberated from a sample of the sodium hydrate solution upon acidification *in vacuo* with sulphuric acid Since the medium was subsequently exposed only momentarily to the atmosphere, the correction applied (a small one) was exact

Oxygen Oxygen was prepared by heating potassium permanganate *in vacuo* and was purified by passing over phosphorus pentoxide and over sodium hydrate (not that purified by alcohol) It was measured dry, the pressure being read on a barometer column Generator, burette and barometer were permanently incorporated in the pump system (fig 2) by fused glass joints The generator was exhausted by the mercury pump and then rinsed out twice with small quantities of oxygen before the gas was generated for use in the work

Control of gaseous environment and collection and analysis of the gases Fermentations were conducted either *in vacuo*[9] or in the presence of gases admitted in known quantities after a vacuum had first been obtained

The fermentation bulbs (fig 1) fitted with stopcocks of a special form described by one of us,[10] were about half or three-quarters filled with cul-

[9] That is, in an atmosphere of water vapor only

[10] Keyes *Science* (N S), xxviii, p 17, 1908

ture medium, and were sterilized After sterilization, the stopcocks of the bulbs were wiped dry with sterile rolls of filter paper and re-greased with sterile lubricant The lubricant was the non-volatile non-antiseptic mixture described by one of us [11] The medium was then inoculated

The bulbs were then freed from gases It was found that this could be done to any desired degree of completion by the use of a good Sprengel water pump, which was capable of maintaining the pressure at from 9 to 15 mm (The use of a mercury pump gave no better results and was moreover unnecessarily troublesome) This method of exhaustion depends upon rinsing out the gas with water vapor In extracting dissolved gases from liquids the driving force becomes less as the process becomes more nearly complete and the process comes practically to an end while detectable amounts of gases are still dissolved, unless by vigorous agitation the liquid is kept well mixed and many new boundary surfaces thus continually formed We found that, without agitation, pumping for an hour after most of the air had been removed was not so efficacious as five or ten minutes' vigorous shaking interrupted by a few exposures to the action of the pump This experience led us to think that the degree of exhaustion is controlled, in cases where the space above the liquid is kept as free from gases as in these experiments, by the thoroughness of agitation The bulbs were therefore subjected alternately to the action of the pump, by opening the stopcock of the bulb for an instant, and to a vigorous shaking This was repeated until a metallic sound was emitted upon striking the bulb hard with the finger ends[12] and was then further continued until this effect (which cannot be produced until the vacuum is rather good) was produced only with great difficulty As the liquid was shaken, falling drops of liquid clicked metallically against the sides of the bulb We believe that we were able to treat the bulbs with practical uniformity and that before we finished the process the gas that remained in any bulb was negligible in quantity so far as our analyses were concerned [13]

If gas was to be admitted, the bulb was now connected to the pump system by a short piece of heavy walled rubber tubing, which was well painted with hot adherent grease Both ends of the tube were bound tightly on with copper wire The tubing connecting the bulb with the gas burette was evacuated by the use of the mercury pump, and the gas, having been measured, was pushed over into the bulb By careful manipulation practically all the gas could be passed over into the bulb without admitting any mercury

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[11] Keyes *Journ Amer Chem Soc*, xxxi, p 1271, 1909

[12] Special experiments have shown us that this sound is occasioned by the tearing apart of the liquid with the liberation of a bubble or two of gas and the consequent collapse of the newly formed walls of liquid Thus the range of concentrations of dissolved gases at which this effect is possible, should be, as it in fact is, limited in both directions

[13] Except possibly in the case of *B typhosus*, where the total gas evolution was very small

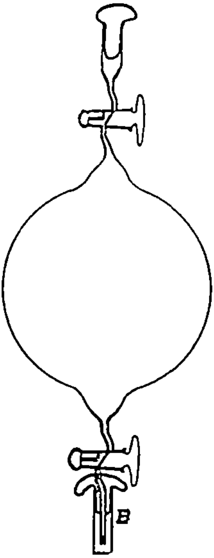

FIG 1 FERMENTATION BULB FITTED WITH VACUUM STOPCOCKS

B, bottle, secured by cotton plug, serving to keep sterile the capillary lead Capacity of bulb, about 300 cc

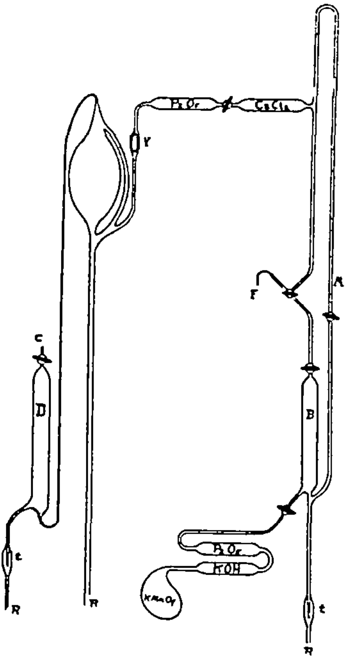

FIG 2 THE PUMP SYSTEM

B, oxygen measuring burette, *D*, delivery tube—it connects with gas analysis apparatus at *C*, *M*, manometer column, provided with meter stick, *t*, *t*, air traps, *V*, valve tight to ascending mercury Fermentation bulb connects at *F* Mercury reservoirs connect by heavy rubber tubing at *R*, *R*, *R*

After incubation at about 37°C, the gases in the bulb were recovered by the mercury pump and analyzed In every instance, the whole obtainable quantity was pumped out and the whole quantity was used for the analysis or else a measured portion after the gases were well mixed The gas absorptions were conducted according to the method of Hempel The burettes were filled with mercury Carbon dioxide was absorbed by strong caustic soda solution (after the total volume had been measured dry), oxygen, by phosphorus or when necessary by alkaline pyrogallol, and hydrogen

was determined by exploding with a quantity of air sufficient to avoid the burning of nitrogen Other gases were absent Sometimes the Hempel pipettes were used for the reagents, but when small amounts of gas were to be analyzed, they were generally measured under diminished pressure, and treated with absorbing reagents in tubes inverted over mercury, as in the method given by Travers [14] Sometimes the diminution of pressure was read on an open manometer, and sometimes the actual pressure was read on a barometer (the space of which was saturated with water vapor) sealed to the burette The work was done in a cellar where the temperature was sufficiently constant All gas volumes have been calculated for 0°C , 760 mm and dryness The medium was always strongly acid after incubation so that no carbon dioxide was retained chemically

Experiments with B Coli

Experiment 1 *B coli* was grown *in vacuo* for 48 2 hours on 250 cc of a medium containing 1 00 per cent each of Witte's peptone and Merck's "Highest Purity" dextrose in distilled water, prepared hot and filtered

Total gas	137 0 cc
CO_2	56 1 per cent
H_2	43 0 per cent
CO_2 per gram of dextrose	30 7 cc
Ratio, CO_2 H_2	1 31

The ratio 1 28 was found by one of us[15] for beef infusion broth containing 1 per cent each of dextrose and Witte's peptone

It seemed better to work with a medium containing no substances of unknown composition One such, described by Dolt,[16] has the advantage that no amino group is present so that retention of hydrogen by the type of reaction found by Harden for asparagine is excluded It consisted of 1 00 per cent ammonium lactate, 1 00 per cent dextrose and 0 200 per cent disodium phosphate in distilled water, prepared without heating In table I are given some experiments upon this medium, arranged according to the length of incubation

The influence of phosphate, of nitrate and of increased dextrose percentage is shown in table II The medium contained 1 00 per

[14] Travers *Study of Gases*, 1901, p 28
[15] Keyes *Journ of Med Res* , xxi (N S xvi), p 69, 1909
[16] Dolt *Journ Inf Dis* , v, p 616, 1908

TABLE I

B coli in vacuo

EXP NO	VOLUME OF MEDIUM	INCUBATION AT 37	TOTAL GAS	CO_2	H_2	CO_2 PER GRAM DEXTROSE	$\frac{CO_2}{H_2}$
	cc	*hours*	*cc*	*per cent*	*per cent*	*cc*	
2	100	23 3	27 60	51 1		14 10	
3	25	40 0	7 21	49 5	48 1	14 30	1 03
4	100	44 3	28 40	51 3	48 1	14 55	1 06
5	100	58 2	28 60	51 1	47 0	14 60	1 09

cent of ammonium lactate and various quantities of dextrose, sodium phosphate and ammonium nitrate, as noted In experiments 7, 8 and 9 Merck's "Highest Purity" dextrose was used, in experiment 6, the same recrystallized from alcohol, in experiment 10, a nitrate-free dextrose (Merck's "Pure") recrystallized

The effect of an increase of phosphate is to increase the total gas formation, probably by delaying acid inhibition, and to increase the formation of carbon dioxide more than hydrogen

The effect of nitrate is to decrease slightly the carbon dioxide formation and to use up most (or all) of the hydrogen that would otherwise be produced The latter effect has been noted by Pakes and Jollyman [17] Merck's "Highest Purity" dextrose was found by the phenolsulphonic acid method to contain 0 04 per cent of sodium nitrate, which could be reduced to 0 01 per cent by recrystallization Assuming proportionality, the result of experiment 9 would indicate that the amount of nitrate present in 1 gram of the "Highest Purity" dextrose could use up 0 012 cc of hydrogen, a quantity which is negligible for *B coli* fermentations but which would be of great significance in *B typhosus* fermentations The ammonium lactate and sodium phosphate were found free from nitrates by the same (controlled) test

In another part of our work, to be communicated in a second paper, we have studied somewhat fully the effect of oxygen upon *B coli* fermentations of dextrose in an asparagine medium The following figures show the results for an aerobic fermentation by *B coli* of a medium composed of 1 00 per cent "Highest Purity"

[17] Pakes and Jollyman *Journ Chem Soc* (Transactions), lxxix, p 386, 1901

TABLE II

B coli in vacuo

EXP NO	COMPOSITION OF MEDIUM*			VOLUME OF MEDIUM	INCUBATION AT 37°	TOTAL GAS	CO_2	H_2	CO_2 PER GRAM DEXTROSE	$\frac{CO_2}{H_2}$
	Dextrose	Na_2HPO_4	$(NH_4)NO_3$							
	per cent	*per cent*	*per cent*	*cc*	*hours*	*cc*	*per cent*	*per cent*	*cc*	
6	1 00	0 10		25	42 8	3 72	48 3	41 9	7 20	1 15
7	1 00	0 60		100	48 2	36 4	63 5		23 10	
8	1 00	0 60		100	52 7	68 3	63 5		43 40	
9	1 00	0 20	0 40	100	72 0	14 0	95 8		13 40	
10	4 00	0 20		50	580	20 4	50 3	47 2	5 12	1 07

* Exclusive of 1 00 per cent ammonium lactate

TABLE III

Gas production of B typhosus in vacuo*

EXP NO	VOLUME OF MEDIUM	INCUBATION AT 37°	TOTAL GAS	CO_2	H_2	RESIDUAL GAS	SUM†	PER GRAM OF DEXTROSE		$\frac{CO_2}{H_2}$
								CO_2	H_2	
	cc	*hours*	*cc*	*per cent*	*per cent*	*per cent*	*per cent*	*cc*	*cc*	
1	213	48 2	3 26	92 4	2 4	5 1	99 9	1 41	0 038	38 5
2	193	72 2	3 40	92 10	1 96	5 98	100 04	1 63	0 033	47 0
3	250	360	5 44	97 10	2 13	0 76	99 99	2 12	0 044	45 6
4	150	384	3 22	93 25	2 03	4 44	99 72	1 99	0 042	46 0
5	150	108	2 01	92 15	4 90	2 47	99 52	1 24	0 066	18 8

* In experiments 1 2 and 5 strain A was used in experiments 3 and 4 strain B

† The figures in this column represent the sums of the analytical percentages recorded in the three preceding columns

dextrose, 1 00 per cent ammonium lactate and 0 100 per cent disodium phosphate

Experiment 11 B Coli

Oxygen admitted	4 59 cc
Incubation at 37°	108 hours
Total gas	10 56 cc
CO_2	5 18 cc
O_2	2 58 cc
H_2	0 96 cc
CO per gram of dextrose	10 36 cc
Ratio, CO H_2	5 4
Oxygen retained	2 01 cc

Experiments 3, 4, 5 and 10 show that it is possible to find conditions such that nearly equal volumes of CO_2 and H_2 are obtained but that the CO_2 is always in slight excess Evidently the presence of oxygen, of nitrates or of sodium phosphate, since all these substances tend to increase the ratio of carbon dioxide to hydrogen, prevents the realization of the value unity for this ratio Dolt[18] found that *B coli* required either phosphates or nitrates for its growth We have found that in the absence of nitrates anaerobic growth vanishes if the phosphate content is sensibly reduced below the lowest concentration used in the experiments given above, so that it appears that, in spite of a systematic error due to the greater solubility of carbon dioxide in the culture liquid, it is not possible to collect equal volumes of carbon dioxide and hydrogen This means that if, as Harden concluded, the decomposition of dextrose by the action of *B coli* results in the formation of an equal number of molecules of carbon dioxide and hydrogen, according to the "classical" fermentation of Duclaux,[19] there occurs also a process which either uses up hydrogen or produces carbon dioxide and which is therefore oxidational This process seems to be necessary for growth

Experiments with B typhosus

A few experiments were made with two strains of the typhoid bacillus Neither strain could be grown on Uschinski's solution, on Fraenkel's modification or on various other simple media

[18] Dolt *Journ Inf Dis*, v, p 616, 1908
[19] Duclaux *Traité de microbiologie*, iv, p 49, 1901

The culture fluid consisted of 1 00 per cent each of Witte's peptone and dextrose in distilled water, prepared hot and filtered In the first four experiments Merck's "Highest Purity" dextrose was used and in the fifth a preparation made by recrystallizing Merck's "Pure" (nitrate-free) dextrose from alcohol

The following analysis for experiment 5 typifies the procedure followed in the analysis of small quantities of gases The explo-

Analysis of experiment 5

	OBSERVED VOLUME	MANOMETER READINGS		PRESSURE	TEMPERATURE	VOLUME AT 760 MM.	MEAN VOLUME
					°C		
Total gas	4 20	523	124	399	22 0	2 150	2 183
	6 80	357	114	243		2 180	
	5 70	413	118	295	22 9	2 220	
After KOH	1 00	264	138	126	22 5	0 166	0 171
	5 00	147	121	26	23 0	0 171	
	2 35	187	132	55		0 170	
	1 80	204	134	70	22 9	0 166	
	1 40	234	135	99		0 182	
Air added	2 60	544	130	414	23 1	1 420	1 458
	3 75	425	126	299		1 470	
	5 15	339	120	219		1 480	
	5 80	309	118	191	23 0	1 460	
	4 60	365	123	242		1 460	
Pass spark	4 60	363	123	244		1 460	
Added electrolytic gas in small quantities until mixture exploded on passing spark	3 20	442	129	313		1 310	1 298
	5 60	296	119	177		1 300	
	6 80	258	114	144		1 290	
	3 40	410	128	282	23 0	1 290	

The result (volumes at room temperature, but dry without correction because the barometer space was saturated with moisture) is therefore

CO_2	2 012	Residual	0 054
H_2	0 107	Sum	2 173

All gas volumes were further reduced to 0°C

sive gas was calculated as hydrogen, and the closeness with which the sums of the analytical percentages thus found approach 100 per cent indicates that the explosive gas was hydrogen On so small a quantity of gas as that remaining for the hydrogen determination the non-production of carbon dioxide upon explosion could not be certainly proved

Since the presence of dextrose is of great importance for growth of bacteria in the absence of oxygen, it is not possible to show by the omission of dextrose what is probably the truth that the gases obtained came from the dextrose Nevertheless a significant amount of fermentable sugar could not have been present in the peptone, since it was found that *B coli* produced anaërobically on a 1 per cent solution of Witte's peptone in forty-eight hours at 37°C only 0 095 cc of CO_2 (38 per cent of the total gas) per gram of peptone, and *B typhosus* produced under the same conditions less than 0 02 cc of CO_2 per gram of peptone

These results show that carbon dioxide and hydrogen are evolved by the action of *B typhosus* on dextrose, but that the amounts produced are very much less than those produced by *B coli*, and the ratio of carbon dioxide to hydrogen is many times higher in the case of *B typhosus* Upon the nitrate-free dextrose (experiment 5) considerably more hydrogen is produced The increase per gram of dextrose is about 0 027 cc, whereas the volume of hydrogen which the amount of nitrate in question can use up in a *B coli* fermentation we have found above to be 0 012 cc

Just as the amount of nitrate present as impurity in the dextrose, while it introduced no significant error in the results for *B coli*, made a large difference in the results for *B typhosus* (where the total amount of gas was much smaller), so may the principles in the peptone which increase the ratio with *B coli* make a greater difference with *B typhosus* It is possible that this is wholly responsible for the difference in the values of the ratio found for the two organisms

Experiments with Bact welchii

Bact welchii was grown anaërobically on a medium consisting of 1 per cent each of Witte's peptone and Merck's "Highest Purity" dextrose in distilled water No alkali was added

TABLE IV

Bact welchii in vacuo

EXP NO	VOLUME OF MEDIUM	INCUBATION AT 37°	TOTAL GAS	CO_2	H_2	RESIDUAL GAS	CO_2 PER GRAM DEXTROSE	$\frac{CO_2}{H_2}$
	cc	*hours*	*cc*	*per cent*	*per cent*	*per cent*	*cc*	
1	100	120	61 3	60 0	39 3	0 5	36 8	1 53
2	50	336	63 5	58 5	41 2	0 3	74 2	1 43

The results differ chiefly from those found for *B coli* upon this medium in the larger volumes of gas found per gram of dextrose

Comparison of the three microörganisms

Some of the foregoing experiments give a basis for a comparison of the three organisms, namely, those anaerobic fermentations of a medium consisting of 1 per cent each of Witte's peptone and dextrose In table V are given the maximum volumes of carbon dioxide evolved per gram of dextrose and the ratio CO_2 H_2 The lower value of the ratio given under *B typhosus* is that obtained with nitrate-free dextrose

TABLE V

	B TYPHOSUS	B COLI	BACT WELCHII
Carbon dioxide in cubic centimeters	2 1	30 7*	74
Ratio, CO_2 H_2	from 19 to 44	1 31	1 48

* 48 2 hours possibly not maximum amount obtainable

SUMMARY

The gas evolution accompanying the growth of certain bacteria on culture media containing dextrose has been studied by an exact method

I The principal results for *B coli* are

A Dextrose-peptone media yield considerably larger volumes of carbon dioxide than of hydrogen upon anaerobic fermentation The volume-ratio CO_2 H_2 is 1 31

B A suitable "synthetic" medium (composed of ammonium lactate, disodium phosphate and dextrose) yields anaërobically nearly equal volumes of the two gases The ratio CO_2 H_2 is always

greater than unity and has a mean value of 1 06 for a medium of given composition

1 The presence of oxygen raises the value of this ratio

2 Increase of phosphate content also raises the value

3 The phosphate cannot be reduced sensibly in quantity, or substituted by a salt less objectionable

4 The value 1 06 for the ratio $CO_2.H_2$ is minimal This means that if the principal gas reaction consists of a liberation of an equal number of molecules of carbon dioxide and hydrogen from dextrose there also occurs an accompanying gas reaction of the nature of an oxidation

II *B typhosus* produces anaerobically from a dextrose-peptone medium small volumes of carbon dioxide and an explosive gas, probably hydrogen The ratio CO_2 H_2 is never lower than 19

III *Bact welchii* produces anaerobically from a dextrose-peptone medium large volumes of carbon dioxide and hydrogen The ratio CO_2 H_2 is 1 48

A CONTRIBUTION TO OUR KNOWLEDGE OF THE GAS METABOLISM OF BACTERIA

SECOND PAPER

THE ABSORPTION OF OXYGEN BY GROWING CULTURES OF B COLI AND OF BACT WELCHII

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(Received for publication, October 9, 1912)

We have studied the gas production of *B coli* and of *Bact welchii* for various incubation periods in the presence of oxygen admitted to the fermentation bulbs in known quantities after a vacuum had first been obtained The technique employed has been described by us in the preceding paper

From the data so obtained we can derive information concerning the rate at which oxygen is absorbed and concerning the relations existing among the quantities of oxygen absorbed and the quantities of carbon dioxide and hydrogen evolved

We have not found in the literature any work on these points for any microorganism where the atmosphere over the culture was accurately controlled and the analyses were made on portions of gas accurately sampled

Experiments with B coli

The culture medium consisted of 1 00 per cent each of Merck's "Highest Purity" dextrose and asparagine and 0 200 per cent of disodium phosphate and was made neutral to phenolphthalein with sodium hydrate Corrections were made for the carbon dioxide thus introduced, as explained in the first paper, and the values of the corrections are given with the analyses

The results of the gas analyses are given in table I

The values of the ratio, CO_2 H_2, vary enormously, and are many times the value previously obtained by one of us[1] for anaërobic fermentations

In an experiment described in the preceding paper (Exp 11, p 299) the retention of oxygen was found to be 2 0 cc, whereas the decrease in yield of hydrogen (*i e*, that amount which, together with the amount actually found, would make the ratio CO_2:H_2 equal to 1 06, the mean value for anaerobic fermentations on the same medium) is 3 9 cc or only 0 1 cc less than that which the oxygen could have oxidized The medium for which this result was obtained was based on ammonium lactate If this result were the rule, it would indicate that the oxygen was almost quantitatively taken up by nascent hydrogen from the dextrose Similar calculations from the data here presented show, however, that this is not the rule (at least in the case of the asparagine medium) but that the missing volumes of hydrogen are sometimes greater and sometimes smaller than twice the volumes of absorbed oxygen

The volume of oxygen absorbed per unit volume of carbon dioxide evolved (the "respiratory quotient") is given The mean value is 0 135, with a probable error of ± 0 02

The relation between the volume of oxygen admitted to the culture, the volume of oxygen not absorbed and the duration of incubation at 37°C can well be seen from a calculation of the values of the expression $\frac{1}{t} \log \frac{V}{v}$, where t is the time in hours, V is the volume of oxygen admitted, and v is the volume of the unabsorbed oxygen Since $\frac{V}{v}$ here equals the ratio of the corresponding pressures, the given expression is that for the constant of a monomolecular gas reaction It is in fact nearly constant The values are given in table III We should note, however, that the fermentation bulbs were not shaken during incubation, so that although a certain degree of agitation was imparted to the medium by the brisk evolution of gas, the rate of oxygen absorption may, nevertheless (namely, if relatively fast), have been limited by the rate of distribution In this case the given expression should be constant

[1] Keyes *Journ of Med Res*, xxi (N S, xvi), p 69, 1909

The data given in table I may properly be compared with those obtained for anaerobic fermentations induced by *B coli* on the same medium, and given by one of us in a previous paper,[2] with the following results (1) Smaller volumes of carbon dioxide are produced aerobically than anaerobically, for all periods of time (2) For the same amount of carbon dioxide, less hydrogen is obtained aerobically than anaerobically The presence of oxygen therefore appears to lessen the production of gases from dextrose and also either to cause some output of carbon dioxide by a respiratory process or to cause a disappearance of hydrogen (presumably) by oxidation

Experiments with Bact welchii

Similar experiments were made with *Bact welchii* The medium consisted of 1 00 per cent each of Witte's peptone and Merck's "Highest Purity" dextrose in distilled water, prepared hot and filtered No alkali was added Other conditions were the same as before, except that all durations of incubation were much longer and the pressures of oxygen were much smaller

The values of the expression $\frac{1}{t} \log \frac{V}{v}$ are given in table III They are very nearly constant and are about one-third the value found for *B coli*

All other results are given in table II The mean value for the respiratory quotient $\left(\frac{O_2}{CO_2}\right)$ is 0 014, *i e*, one-tenth the value found for *B coli*, with a probable error of $\pm$ 0 002

The ratio CO_2 H_2 is slightly raised by the presence of oxygen,[3] and does not vary for this organism in great degree as it does for *B coli*

As with *B coli*, the "missing" volumes of hydrogen are not equal to twice the volumes of oxygen absorbed but are sometimes greater and sometimes smaller It is perhaps of significance in this connection that both the media used for these organisms permit side-reactions which prevent the evolution of equal volumes of carbon dioxide and hydrogen by anaerobic fermentation of dextrose, as discussed in the first paper of this study

[2] Keyes *loc cit*

[3] Values for anaërobic fermentations are given in the first paper of this series

TABLE I

B coli

All gas volumes are reduced to standard conditions 0°C , 760 mm of mercury and to dryness

NUMBER	WEIGHT MEDIUM	CORRECTION FOR CO_2 IN NaOH	SPACE ABOVE LIQUID	O_2 ADDED	INITIAL PRESSURE of O_2 in MM Hg (APPROX)	INCUBATION AT 37 C	TOTAL GAS FOUND	CO_2 FOUND	CO_2 CORR FOR NaOH	O_2 FOUND	H_2 FOUND	N_2 FOUND	VOLUME RATIO $\frac{CO_2}{H_2}$	O_2 ABSORBED	RESPIR QUOTIENT $\frac{O_2}{CO_2}$	CO_2 PER GRAM DEXTROSE	CO_2 PER GRAM DEXTROSE ANAEROBICALLY (EXPERIMENTS OF KEYES 09)
	grams	*cc*	*cc*	*cc*		*hours*	*cc*	*cc*	*cc*	*cc*	*cc*	*cc*		*cc*		*cc*	*cc*
1	200	0 78	50	5 27	80	24	28 61	24 52	23 74	3 81	0 04	0 24	590 0	1 46	0 062	11 87	16 9
2	135	0 00	115	5 34	35	66	20 06	14 79	14 79	3 40	1 74	N D†	8 6	1 94	0 132	10 95	29 −63
3	241	0 88	154	5 28	26	72	36 52	25 75	24 87	2 74	7 38	0 75	3 4	2 54	0 102	10 32	29 −63
4	112	0 46	183	10 36	43	74	29 82	19 65	19 19	5 35	4 52	0 30	4 2	5 01	0 261	17 12	29 −63
5	200	0 78	50	5 05	76	119	37 47	34 11	33 33	2 68	0 17	0 52	196 0	2 37	0 071	16 67	63 4+
6	180	0 57	70	7 38	80	167	48 97	45 94	45 37	2 29	0 47	0 33	96 5	5 09	0 112	25 20	63 4+
7	240	0 85	55	5 97	82	192	27 07	21 33	20 48	1 79	2 44	1 44	8 4	4 18	0 204	8 36	63 4+
															Mean = 0 135 ± 0 02		

In anaerobic fermentations this ratio does not change with the time and has the mean value 1 77 (Keyes *Journ Amer Chem Soc* xxxi p 1271)

† N D = Not done

TABLE II

Bact welchii

All gas volumes are reduced to standard conditions

NUMBER	WEIGHT MEDIUM	SPACE ABOVE LIQUID	O_2 ADDED	INITIAL PRESSURE of O_2 in MM Hg (APPROX)	INCUBATION AT 37 C	TOTAL GAS FOUND	CO_2 FOUND	O_2 FOUND	H_2 FOUND	N_2 FOUND	VOLUME RATIO $\frac{CO_2}{H_2}$	O_2 ABSORBED	RESPIR QUOTIENT $\frac{O_2}{CO_2}$	CO_2 PER GRAM DEXTROSE	CO_2 PER GRAM DEXTROSE ANAËROBICALLY (FIRST PAPER OF THIS STUDY)
	grams	*cc*	*cc*		*hours*	*cc*	*cc*	*cc*	*cc*	*cc*		*cc*		*cc*	*cc*
1	63 0	237	0 38	0 12	240	73 28	43 35	0 18	28 38	0 42	1 53	0 19	0 0044	68 80	
2	57 5	242	0 97	3 05	240	57 07	36 06	0 55	19 55	0 90	1 85	0 42	0 0116	62 80	
3	42 5	253	0 94	2 85	336	54 36	33 20	0 65	19 84	0 61	1 67	0 29	0 0088	78 20	74 20
4	52 0	248	0 73	2 25	384	65 14	38 20	0 28	26 53	N D †	1 44	0 45	0 0118	73 50	
5	55 0	245	1 24	3 85	432	71 92	45 01	0 28	25 72	0 72	1 75	0 96	0 0214	81 90	
6	49 0	251	1 62	3 90	480	71 55	45 45	0 37	25 20	0 55	1 80	1 25	0 0275	92 80	
													Mean =0 014 ±0 002		

For anaërobic fermentations the values for this ratio were 1 53 and 1 42 as found in the preceding paper

† N D —Not done

TABLE III

Values of $\frac{1}{t} \log \frac{\text{volume } O_2 \text{ admitted}}{\text{volume } O_2 \text{ recovered}}$

t = time in hours, common logarithms

HOURS	B COLI	HOURS	BACT WELCHII
24	0 0059	240	0 0013
66	0 0030	240	0 0010
72	0 0040	336	0 0005
74	0 0039	384	0 0011
119	0 0023	432	0 0015
167	0 0030	480	0 0013
192	0 0027		
Mean value	0 0035 ± 0 0003		0 0011 ± 0 0001

SUMMARY

The absorption of oxygen by growing cultures on dextrose media of *B coli* and of (the strict anaerobe) *Bact welchii* has been studied by an exact method Data afforded by complete gas analyses are given The following comparisons are made possible

1 For both microorganisms, the absorption of oxygen simulates a monomolecular reaction

2 The mean values of the respiratory quotients, although the probable error of each is large, are widely different for the two microorganisms

3 With varying pressures of oxygen, the ratio CO_2 H_2 varies enormously in the case of *B coli*, but varies only slightly in the case of *Bact welchii*

We think that the numerical differences in the results for the two microorganisms may *possibly* be referable to differences in experimental conditions, if so, probably to the differences in oxygen pressures

THE ORGANIC PHOSPHORIC ACID OF COTTON SEED MEAL

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(Received for publication, October 12, 1912)

INTRODUCTION

In the investigation of the organic phosphoric acids present in various cereals and feeding stuffs which is being carried out in this laboratory, cotton seed meal was also examined Earlier work by other investigators has shown that this product probably contains some complex organic phosphoric acid[1] It seems, however, that if such a substance is present it has not been isolated in pure form nor have its properties been fully studied

The opinion seems to be generally held that cotton seed meal contains some poisonous principle, but the exact nature of this principle has never been definitely determined It has been claimed that pyro- and metaphosphoric acids were present in cotton seed meal[2] and it was thought that the poisonous properties of the product were due to the presence of salts of these acids More recent work by Crawford[3] led him to believe that the poisonous principle was a salt of either a simple inorganic or a complex organic pyrophosphoric acid

The presence of these acids has been adduced from the fact that extracts of cotton seed meal give similar reactions to the above acids, viz , anomalous behavior towards ammonium molybdate, white precipitates with silver nitrate and coagulation

[1] Rather Texas Agric Exp Station, Bulletin 146, 1912

[2] Hardin South Carolina Agric Exp Station, Bulletin 8 (new series), 1892

[3] Crawford *Journ of Pharmacol and Exp Therapeutics*, i 1910

of egg albumen, further, the poisonous effects resemble those given by these acids Aside from these reactions, however, there is no proof whatever that either pyro- or metaphosphoric acid is present in cotton seed meal

The purpose of the present investigation was to isolate and identify, if possible, the organic phosphoric acid in cotton seed meal We are, consequently, unable either to deny or affirm the absence or presence of pyro- or metaphosphoric acid in this product We have found, however, that the organic phosphoric acid isolated from cotton seed meal gives all the reactions reported by the above authors, which they considered as evidence of the presence of pyro- and metaphosphoric acid It seems, therefore, probable that the reactions referred to are due to the organic phosphoric acid rather than to pyro- or metaphosphoric acids

The preparation of the substance and its purification will be more fully described in the experimental part It will suffice to state here that the cotton seed meal was extracted with 0 2 per cent hydrochloric acid and the substance isolated as the barium salt The purification of the substance is very difficult The extract contains large quantities of soluble impurities, mucilaginous substances, proteins, etc , which render the purification extremely difficult and tedious In addition to the above, there is apparently some carbohydrate associated with the organic phosphoric acid, the removal of which requires much time For the same reasons the yield of the pure product is very unsatisfactory

The compound finally obtained is very similar to phytic acid so far as composition and reactions are concerned In fact it is impossible to determine from the present data whether the substance is phytic acid or an isomer Both yield inosite when heated in a sealed tube with dilute sulphuric acid and the reactions of aqueous soultions of the free acids can hardly be differentiated The most striking difference is that the barium salt of the product from cotton seed meal shows a decided tendency to crystallize, a property which we have never observed when working with barium phytate under the same conditions

If the substance from cotton seed meal is precipitated from acid solutions with barium hydroxide it separates as a white

amorphous precipitate When the dried precipitate is digested in 0 5 per cent hydrochloric acid it dissolves very readily but after a few minutes it precipitates again Under the microscope this precipitate is seen to consist of balls or globular masses of very fine needle-shaped crystals The dilute hydrochloric acid solution of the barium salt gives a white amorphous precipitate on the addition of alcohol, on standing for several hours, however, it slowly assumes the same crystalline form as mentioned above The free acid is not precipitated by barium chloride but if such a solution is allowed to stand over night or longer the barium salt will separate in fine needle-shaped crystals, grouped in the same general form as above but the individual crystals are much larger The amorphous precipitates are very soluble in 0 5 per cent hydrochloric acid but, after the substance has assumed the crystalline form, it is very slightly soluble in this medium

While the barium salt was easily obtained in crystalline form it did not contain a constant amount of the base The variations would sometimes amount to as much as 3 or 4 per cent, depending upon the amount of the base present in the solution and the conditions under which the substance separated In the presence of a large excess of barium chloride a salt corresponding nearly to tetrabarium phytate crystallizes out, when a small amount of barium chloride is present salts showing the above mentioned variations are formed, but when the substance has been repeatedly separated from acid solutions with alcohol a salt is obtained which corresponds nearly to tribarium phytate

The aqueous solution of the free acid gives a heavy white amorphous precipitate with excess of silver nitrate, with ammonium molybdate a heavy white crystalline precipitate is produced which remains unchanged in the cold for a long time but when heated soon turns yellowish in color These reactions are identical with those given by phytic acid, with other metals both acids give apparently identical reactions

The dilute aqueous solution of the acid coagulates egg albumen at once This property of coagulating egg albumen, however, is not peculiar to the acid from cotton seed meal Phytic acid found to produce an identical effect The tetraphosphoric

nium molybdate but after continued heating a slight precipitate was obtained The substance was free from nitrogen and sulphur

After drying at 105° in vacuum over phosphorus pentoxide it was analyzed

0 2925 gram substance gave 0 0894 gram H_2O and 0 2338 gram CO_2
0 2514 gram substance gave 0 0972 gram $BaSO_4$ and 0 0933 gram $Mg_2P_2O_7$
Found C = 21 80, H = 3 42, P = 10 34, Ba = 22 75 per cent

While the substance was very slightly soluble in boiling water it was found when it was rubbed up in a mortar with a small quantity of cold water that it quickly dissolved but it began soon to separate again Under the microscope the precipitate was seen to consist of small balls or globular masses of very fine microscopic needles

Four grams of the substance were treated as mentioned above After standing for two days at room temperature the crystalline precipitate was filtered off, washed in water, alcohol and ether and dried in the air The snow-white crystalline powder was analyzed after previous drying at 105° in vacuum over phosphorus pentoxide

0 2092 gram substance lost 0 0291 gram H_2O
0 1801 gram substance gave 0 0232 gram H_2O and 0 0379 gram CO_2
0 1754 gram substance gave 0 1262 gram $BaSO_4$ and 0 0965 gram $Mg_2P_2O_7$
Found C = 5 73, H = 1 44, P = 15 33, Ba = 42 34, H_2O = 13 91 per cent

The composition of this product differs entirely from that of the starting material but it agrees closely with that required for tetrabarium phytate

Calculated for tetrabarium phytate, $C_6H_{16}O_{27}P_6Ba_4$ = 1255
C = 5 73, H = 1 27, P = 14 82, Ba = 43 74, 11 H_2O = 13 62 per cent

The filtrate from the above crystalline compound was precipitated by alcohol, filtered, washed and dried in vacuum over sulphuric acid It was a perfectly white amorphous powder It was analyzed after drying at 105° in vacuum over phosphorus pentoxide

0 1758 gram substance gave 0 0790 gram H_2O and 0 2222 gram CO_2
0 1247 gram substance gave 0 0223 gram $BaSO_4$ and 0 0201 gram $Mg_2P_2O_7$
Found C = 34 47, H = 5 02, P = 4 49, Ba = 10 52 per cent

The substance was very soluble in 0 5 per cent hydrochloric

acid, in which it gave the same thick, mucilaginous, slightly opalescent solution as mentioned above

The compound first analyzed is evidently not homogeneous It apparently consists of some carbohydrate or gummy substance and an organic phosphorus compound, the latter crystallizes from the aqueous solution in nearly pure form but the substances cannot be separated by precipitating the dilute acid solutions with alcohol This gummy substance has not been isolated in pure form and we are entirely in the dark as to its nature and composition

A portion of the above crystalline barium salt was used for the preparation of the free acid The substance was, however, not pure and it had not been washed free of the mother liquor The acid was prepared by the usual method, that is, the barium salt was decomposed with a slight excess of sulphuric acid, the filtered solution precipitated with copper acetate, the latter filtered, washed and decomposed with hydrogen sulphide, again filtered and the filtrate evaporated in vacuum at a temperature of 40–45° and finally dried in vacuum over sulphuric acid In appearance and reactions the acid was practically identical with phytic acid except that after boiling with dilute hydrochloric acid and neutralizing it slightly reduced Fehling's solution This reduction, however, we believe to be due to admixed impurities for, as stated above, the acid was not prepared from a pure compound

The aqueous solution of the acid coagulates egg albumen at once As has been already mentioned phytic acid gives the same reaction, as well as the inosite esters of phosphoric and pyrophosphoric acids Apparently, therefore, no special significance can be attached to this reaction

The acid gave the following result on analysis after previously drying at 105° in vacuum over phosphorus pentoxide

Found C = 11 71, H = 3 07, P = 26 35 per cent

The crystalline precipitate mentioned on page 315, which separated from the solution of the first barium precipitate in 0 5 per cent hydrochloric acid, was treated with about 5 per cent hydrochloric acid in which the greater portion dissolved The insoluble matter was removed by centrifuging and the solution precipitated with alcohol This operation was repeated a second time when

the substance was obtained nearly white It differed from the first preparation in that its solution in dilute hydrochloric acid was neither mucilaginous nor opalescent For further purification the substance was first precipitated by barium hydroxide from its dilute hydrochloric acid solution and then twice precipitated from dilute hydrochloric acid with alcohol The precipitates produced by the alcohol were amorphous at first but when allowed to stand over night in the mother liquor they always changed to the same crystalline form as previously mentioned

After precipitating the last time with alcohol the substance was quickly filtered, washed in dilute alcohol, alcohol and ether and dried in vacuum over sulphuric acid The product was a snow-white amorphous powder and it weighed 7 4 grams

The filtrate from the above was allowed to stand over night when a small amount of the substance crystallized out The crystals were filtered, washed in dilute alcohol, alcohol and ether and dried in the air The substance was free from chlorine and gave no appreciable color reaction with phloroglucine or orcine For analysis it was dried at 105° in vacuum over phosphorus pentoxide

0 2450 gram substance lost 0 0272 gram H_2O on drying
0 2178 gram substance gave 0 0284 gram H_2O and 0 0522 gram CO_2
0 1776 gram substance gave 0 1170 gram $BaSO_4$ and 0 1078 gram $Mg_2P_2O_7$
Found C = 6 53, H = 1 45, P = 16 91, Ba = 38 76, H_2O = 11 10 per cent

This substance agrees nearly in composition with tribarium phytate

Calculated for tribarium phytate, $C_6H_{18}O_{27}P_6Ba_3$ = 1120
C = 6 42, H = 1 60, P = 16 60, Ba = 36 78, 8H O = 11 39 per cent

The amorphous product (7 4 grams) mentioned above was analyzed after previous drying at 105° in vacuum over phosphorus pentoxide and the following results obtained

C = 8 04, H = 1 62, P = 16 65, Ba = 36 55 per cent

The substance was free from chlorine It was very slightly soluble in boiling water With phloroglucine it gave a cherry red color, with orcine only a faintly greenish color was produced After boiling in dilute hydrochloric acid, precipitating the barium with sulphuric acid, filtering and neutralizing, it reduced Fehling's solution slightly on boiling Evidently some carbohydrate was still present

For further purification the substance was dissolved in 0.5 per cent hydrochloric acid, filtered and alcohol added until a faint permanent turbidity remained. This was cleared up by the addition of a few drops of dilute hydrochloric acid and the solution allowed to stand at room temperature. The substance soon began to separate in the same crystalline form as before. After standing for two days the crystalline substance was filtered off, washed in water, alcohol and ether and dried in vacuum over sulphuric acid.

The mother liquor was diluted with more alcohol and allowed to stand as before when a further quantity of the same shaped crystals was obtained. After filtering, washing and drying as before these salts were analyzed after first drying at 105°.

Found (first crop of crystals) C = 7.06, H = 1.53, P = 16.46, Ba = 38.16 per cent

Found (second crop of crystals) C = 7.47, H = 1.58, P = 16.46, Ba = 38.12 per cent

In order to determine if further treatment would alter the composition, the whole substance was digested in 50 per cent acetic acid over night, filtered, washed in water, alcohol and ether and dried in the desiccator. It was then dissolved in 0.5 per cent hydrochloric acid, filtered and the solution brought to crystallization by the careful addition of alcohol as before. The product finally obtained weighed 3.8 grams and it was a snow-white crystalline powder. For analysis it was dried at 105° in vacuum over phosphorus pentoxide.

Found C = 7.10, H = 1.52, P = 17.17, Ba = 38.11 per cent

As continued treatment did not alter the composition and as it separated in crystalline form it was undoubtedly a homogeneous compound.

The free acid prepared from this purified barium salt by the same method as before gave the following result on analysis after previous drying at 78° in vacuum over phosphorus pentoxide.

0.2626 gram substance gave 0.0763 gram H_2O and 0.1049 gram CO_2

0.1733 gram substance gave 0.1686 gram $Mg_2P_2O_7$

Found C = 10.89, H = 3.25, P = 27.11 per cent

Calculated for phytic acid, $C_6H_{24}O_{27}P_6$ = 714

C = 10.08, H = 3.36, P = 26.05 per cent

This acid gave the same reactions as previously described.

Preparation of inosite from the above barium salt

Of the dry salt, 1 34 grams were heated in a sealed tube with 10 cc of 5N sulphuric acid to 160° for about three hours After precipitating with barium hydroxide the inosite was isolated in the usual way and recrystallized from dilute alcohol with addition of ether The product was obtained in colorless needles free from water of crystallization The yield was 0 17 gram or about 77 per cent of the theory It gave the reaction of Scherer and melted at 221° (uncorrected) The air-dried substance was analyzed

Found C = 39 81, H = 6 96 per cent

A further quantity of the barium salt was prepared by the following method which was found to be much less laborious than that used at first The cotton seed meal, 8 kilograms, was digested in 16 liters of 0 2 per cent hydrochloric acid for about five hours It was then pressed through cheese-cloth and the extract filtered through absorbent cotton The extract was precipitated with excess of barium hydroxide, allowed to settle and then centrifuged The precipitate was digested in several liters of 0 5 per cent hydrochloric acid and again centrifuged The free acid was then nearly neutralized with barium hydroxide The precipitate which separated was the barium salt of the organic phosphoric acid This was filtered and treated with 0 5 per cent hydrochloric acid in which it was readily soluble at first but it soon separated in the usual crystal aggregates This was filtered and washed and dissolved in sufficient dilute hydrochloric acid and again filtered The practically colorless filtrate was precipitated by alcohol After filtering it was again dissolved in dilute hydrochloric acid and precipitated with barium hydroxide, filtered and washed in water It was then dissolved in dilute hydrochloric acid, precipitated with alcohol, filtered, washed in dilute alcohol, alcohol and ether and dried in vacuum over sulphuric acid The product was a snow-white amorphous powder and it weighed 24 grams It was dissolved in about 300 cc of 0 5 per cent hydrochloric acid, filtered and allowed to stand a short time when a portion crystallized out This was filtered off, washed several times in water and finally in alcohol and ether and dried in the air The white crystalline powder weighed 7 4

grams The filtrate and washings from above were united and precipitated by alcohol After standing over night the amorphous precipitate had changed to the usual crystalline form After filtering, washing and drying in vacuum over sulphuric acid it weighed 14 7 grams

The above salts were free from chlorine The nitric acid solutions gave no immediate reaction with ammonium molybdate No appreciable color reactions were obtained with phloroglucine or orcine and they did not reduce Fehling's solution Metals other than barium were absent For analysis the substances were dried at 105° in vacuum over phosphorus pentoxide

The first crystalline compound gave the following

$$C = 6\ 05, H = 1\ 45, P = 16\ 51, Ba = 40\ 04, H_2O = 12\ 06 \text{ per cent}$$

This salt is evidently a mixture of the tri- and tetrabarium salt It was recrystallized as follows One gram of the substance was dissolved in about 150 cc of 0 5 per cent hydrochloric acid and the free acid nearly neutralized with barium hydroxide About 0 5 gram of barium chloride was then added and the solution allowed to stand for two days at room temperature The substance separated slowly in the same general crystal form as before except that the individual crystals were much larger These were filtered, washed in water, alcohol and ether and dried in the air Under the microscope the substance appeared homogeneous Yield, 0 9 gram After drying at 105° in vacuum it was analyzed

0 4067 gram substance lost 0 0496 gram on drying
0 3571 gram substance gave 0 0358 gram H_2O and 0 0699 gram CO_2
0 2068 gram substance gave 0 1530 gram $BaSO_4$ and 0 1160 gram $Mg_2P\ O_7$
Found C = 5 33, H = 1 12, P = 15 63, Ba = 43 53, H O = 12 19 per cent
Calculated for tetrabarium phytate $C_6H_{16}O_{27}P_6Ba_4$ = 1255
C = 5 73 H = 1 27, P = 14 82, Ba = 43 74, 10 H O = 12 54 per cent

The second crystalline compound mentioned above gave the following result on analysis

C = 6 88, H = 1 50, P = 15 94, Ba = 37 38 per cent
P = 16 28, Ba = 37 21 per cent

This corresponds to a tribarium salt

A further quantity of the free acid was prepared from this salt by the usual method From 7 grams of the substance practically the theoretical quantity of acid was obtained After drying at 100° the substance was analyzed

0 2319 gram substance gave 0 0767 gram H_2O and 0 0925 gram CO_2

After drying over boiling chloroform over phosphorus pentoxide

0 2378 gram substance gave 0 0703 gram H_2O and 0 0961 gram CO_2
0 1495 gram substance gave 0 1414 gram $Mg_2P_2O_7$
Found C = 10 87, H = 3 70 per cent
C = 11 02, H = 3 30, P = 26 36 per cent
Calculated for phytic acid, $C_6H_{24}O_{27}P_6$ = 714
C = 10 08, H = 3 36, P = 26 05 per cent

This preparation gave the same reactions as those previously mentioned When carefully prepared the acid is a thick colorless syrup readily soluble in water and alcohol Attempts were made to prepare crystalline salts of the acid with organic bases like pyridine and brucine but without success These salts could not be obtained in crystalline form In every case they separated as thick liquids which could not be brought to crystallize even after long standing

The reaction of the aqueous solution of the acid with inorganic bases may be briefly stated as follows

Ammonium molybdate gives a heavy, white, crystalline precipitate Silver nitrate in excess produces a heavy, white, amorphous precipitate Magnesia mixture also gives a voluminous, white, amorphous precipitate It is not precipitated by the chlorides of the alkaline earths but the acetates and hydroxides produce white amorphous precipitates While barium chloride does not give any precipitate if the solution is allowed to stand at room temperature over night or longer the barium salt crystallizes out in delicate, needle-shaped crystals In shape and arrangement these crystals are identical with those previously referred to but they are much larger It immediately coagulates egg albumen A neutral solution of the acid does not reduce Fehling's solution, even after boiling with dilute hydrochloric acid for some time no reduction takes place No appreciable color reaction is given with phloroglucine or orcine

Ferric chloride gives a white precipitate very sparingly soluble in hydrochloric acid Copper acetate in excess gives a bluish-white precipitate

On drying at 78° or 100° the substance turns very dark in color but on drying at 60° the color changes but slightly All the barium salts obtained were strongly acid in reaction on moist litmus paper

It is evident that the substance isolated from cotton seed meal is very similar to phytin The various salts which have been analyzed show but little difference in composition as compared with the corresponding phytin derivatives It may be noted, however, that the analytical results of the purified, so-called tribarium salts point to the empirical formula $C_2H_4P_2O_8Ba$ Such a compound might be a monobasic acid of the formula CH_3PO_4 but it is also isomeric with inosite hexaphosphate and accordingly differs very little in composition from phytic acid

If the organic phosphoric acid in cotton seed meal is identical with phytin, if it is an isomer or if it is a somewhat differently constituted substance can hardly be determined from the data presented in this paper The investigation will be continued

STUDIES IN THE BLOOD RELATIONSHIP OF ANIMALS AS DISPLAYED IN THE COMPOSITION OF THE SERUM PROTEINS I

A COMPARISON OF THE SERA OF THE HORSE, RABBIT, RAT AND OX WITH RESPECT TO THEIR CONTENT OF VARIOUS PROTEINS IN THE NORMAL AND IN THE FASTING CONDITION

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(Received for publication, October 12, 1912)

I METHODS AND RESULTS

A Methods

I have shown in a previous communication[1] that it is possible, with a very fair and a thoroughly definite degree of accuracy, to determine the total quantity and relative proportion of each of three groups of serum proteins by a refractometric method, only employing very small quantities (not necessarily exceeding 20 cc) of serum The method, briefly recapitulated with slight modifications indicated by further experience, is as follows

1 An accurately measured volume, usually 10 cc , of fresh whipped and centrifugalized serum is diluted to ten times its volume with distilled water Carbon dioxide is bubbled through this solution at a good rate (two or three bubbles per second) for at least one hour The precipitate which is thus obtained is allowed to settle for from twelve to sixteen hours The supernatant fluid is then carefully decanted and centrifuged in two 50 cc centrifuge tubes to throw down any flocculi carried over in decantation Meanwhile, the glass cylinder containing the precipitate is filled up to the 100 cc mark with distilled water and the contents thoroughly mixed by shaking A few minutes' energetic centrifugalization of the mother-liquor suffices to throw down any traces of globulin which it contains and the fluid is then

[1] T Brailsford Robertson this *Journal*, xi, p 179, 1912

decanted from the centrifuge tubes, leaving the precipitate closely packed at the bottom. These tubes are then filled with the suspension of globulin in distilled water, the last traces of globulin being washed out of the cylinder with a few cubic centimeters of distilled water. These washings are added to the contents of the centrifuge tubes. The suspension is now energetically centrifuged and in a few minutes the precipitate is found to be tightly packed at the bottom of the tubes. The fluid is now poured off, 1 cc. of $\frac{N}{10}$ KOH or NaOH is added to the contents of one of the tubes and 2 or 3 cc. of distilled water. As soon as all of the globulin is dissolved in the first tube, the contents are transferred to the second tube, the first tube being carefully rinsed with 3 or 4 cc. of distilled water. As soon as the globulin in the second tube is completely dissolved, the contents and rinsings from this tube are transferred to a narrow calibrated[2] measuring cylinder, which is then filled by the addition of distilled water to the 10 cc. mark, that is, to the volume originally occupied by the serum from which the globulin was precipitated.[3] The refractive index of this solution and that of $\frac{N}{100}$ KOH or NaOH are determined at the same temperature. The difference between the two readings, divided by 0.00229, yields *the percentage of "insoluble" globulin in the original serum.*

2. To an accurately measured volume of the same serum is added an equal volume of saturated ammonium sulphate solution and the globulins thus precipitated are filtered off, the filtrate is collected and diluted to one-half with water and the refractive index of the mixture thus obtained is measured. At the same time we measure the refractive index of a one-fourth saturated solution of ammonium sulphate prepared by adding to a portion of the saturated ammonium sulphate solution an equal volume of distilled water and diluting the solution thus obtained to one-half. The difference between these two readings, multiplied by 4 and then diminished by 0.00157 (the refractivity of the non-protein constituents of serum) yields the *total refractivity* of the *albumins of the serum.* If crystallizable albumin be absent,[4] then this figure divided by 0.00177 yields *the percentage of albumin in the original serum.*

3. The refractive index of the original serum is determined and that of $\frac{N}{7}$ NaCl. The difference between the two readings *yields the total refrac-*

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[2] Calibrated against the pipette employed to measure out the original volume of serum.

[3] The centrifugalization of the globulin saves time and also prevents loss in decantation. If, however, the globulin be centrifuged immediately after the passage of the CO_2, it carries down any haemoglobin which the serum may contain and this color cannot be washed out of it again. If, on the contrary, it be allowed to stand for some hours to flocculate it comes down uncolored even from serum tinged with haemoglobin. This fact would appear to indicate danger of contamination by "occlusion," if the precipitate be immediately centrifuged, and the results obtained might conceivably be too high. Hence the procedure indicated above is preferred.

[4] Cf. below.

tivity of the proteins in the serum Subtracting the refractivity of the albumins determined above, we obtain *the total refractivity of the globulins in the serum* This estimate divided by 0 00229 yields *the total percentage of globulins in the serum*

4 The sum of the percentage of albumin and globulin yields *the total percentage of proteins in the serum* If desired this estimate may be checked by a Kjeldahl determination of the nitrogen in the total coagulable protein contained in a measured sample of the serum employed

According to Reiss[5] the refractivity of crystallized serum albumin differs considerably from that of amorphous serum albumin, 1 gram of the former per 100 cc of solvent producing a change of 0 00201 in its refractive index while 1 gram of amorphous albumin per 100 cc of solvent produces a change of 0 00177 in its refractive index If appreciable quantities of crystallizable serum albumin exist as such in a sample of serum under investigation we should expect to detect its presence by a lack of agreement between the percentage of total proteins, determined refractometrically, and the percentage of total proteins determined by coagulation and a Kjeldahl estimate of the total nitrogen in the coagulum As we shall see, however, even when serum known to yield large amounts of crystalline albumin to appropriate treatment is employed, to wit, horse serum, no such lack of agreement can be detected Consequently the method of analysis outlined above yields reliable results whether the serum under examination readily yields crystalline albumin or not

B Results obtained with horse serum

Through the kindness of my colleagues, Drs C M Haring and C L Roadhouse, to whom my sincere thanks are due, I have been able to obtain horse serum from an adult animal which had been deprived of food for about twenty-four hours previous to the bleeding[6] The blood was collected in Erlenmeyer flasks, directly from the jugular vein, defibrinated by shaking with glass beads and immediately centrifuged The clear serum was pipetted off and analyzed immediately

[5] E Reiss *Beitr z chem Physiol u Path*, iv, p 150, 1903, *Arch f exp Path u Pharm*, li, p 118, 1903

[6] Cf under sections C and D regarding the influence of fasting upon the composition of the serum-proteins

The "insoluble" globulin was determined in two 10 cc samples, the albumins were determined in two 5 cc samples In each case the different samples yielded identical results In these and in all of the other determinations reported in this article a Pulfrich refractometer, *reading to within 1′ of the angle of total reflection*, was employed A sodium flame was the source of light The following were the results obtained

TABLE 1 *Horse serum*

	per cent
"Insoluble" globulin	0 34 ± 0 04
Total globulins	3 5 ± 0 15
Total albumins	4 6 ± 0 2
Total proteins	8 1 ± 0 2

The figure following the ± sign is the possible error in the determination due to a possible error of ± 1′ in reading the angle of total reflection [7]

Horse serum, as is well known,[8] readily yields crystalline albumin upon appropriate treatment, accordingly I expected to find a divergence between the percentage of total protein as determined above and the percentage of total proteins estimated by coagulation and the determination of the nitrogen in the coagulum No such divergence was found, however

The determination of the total coagulable protein was carried out as follows, three separate 5 cc samples of the serum being employed

A 9 cm S & S 589 "blue band" filter was about three-fourths filled with absolute alcohol and fresh alcohol was kept dripping into the filter from a burette as rapidly as it passed through A 5 cc sample of the serum was then delivered very slowly, drop by drop, into the alcohol, which passed through quite clear and devoid of precipitate The filter and contained coagulum were dried at 36° over H_2SO_4 for forty-eight hours The nitrogen in the filter and contained precipitate was then determined by the U S

[7] In my previous communication, referred to above, the possible error in determining the total proteins and the albumins is erroneously stated to be ± 0 1 per cent instead of ± 0 2 per cent, while the possible error in determining the total globulin is erroneously stated to be ± 0 1 per cent instead of ± 0 15 per cent

[8] Cf Fr N Schulz *Die Krystallisation von Eiweissstoffen*, Jena, 1901, p 13

official Kjeldahl method [9] The nitrogen was multiplied by 6 29 in estimating the protein The following were the results obtained

TABLE 2 *Alcohol-coagulable protein in horse serum*

SAMPLE	N IN SAMPLE	PER CENT OF ALCOHOL-COAGULABLE PROTEIN IN THE SERUM
	mgm	
1	64 8	8 15
2	62 8	7 90
3	63 3	7 97
		Average 8 00

Hence, within the experimental error of the determinations, the percentage of alcohol-coagulable protein in horse serum is identical with the percentage of total proteins determined on the assumption that all of the albumins in the serum have a refractivity per gram per 100 cc of solvent of 0 00177 It appears, therefore, that albumin of a refractivity of 0 00201 per gram per 100 cc of solvent is not present in sufficient amount in horse serum to appreciably affect the accuracy of refractometric determinations carried out as outlined above The reason for this is readily perceived when we consider the magnitude of the divergence between the above estimates which we should expect to find were the *whole* of the serum albumin of the crystallizable variety In this event, it appears, from the refractometric observations, that in the serum under examination the percentage of total albumin would be 4 1 and the percentage of total proteins, 7 6 No less than 40 per cent of the total albumins would have to be of the crystallizable variety, therefore, in order to cause a divergence of 0 2 per cent between the two estimates of the total proteins Now, as we have seen, the possible error in the refractometric determination of the percentage of total proteins is ± 0 2 per cent Hence we may draw the following conclusions

(1) Horse serum does not contain preformed crystallizable albumin to the extent of over 40 per cent of the total albumins

(2) The refractometric method cannot be employed to determine more precisely than this the quantity of crystallizable albumin contained in sera

[9] U S Department of Agric , Bureau of Chemistry, Bulletin 107, p 5, 1910

(3) Provided not over 40 per cent of the albumins are of the crystallizable variety no appreciable error is introduced into the refractometric determinations of the total albumins and total proteins in sera by assuming that all of the albumins present have a refractivity of 0 00177 per gram per 100 cc of solvent

Expressing the quantity of each of the proteins in the horse serum employed in terms of the percentage of the total proteins which they represent, the above results may be summarized as follows

"Insoluble" globulin	4 2	(± 0 4)
Total globulins	43	(± 2)
Total albumins	57	(± 2)

The figures in brackets represent the plus or minus error in the estimation of these percentages which would be brought about by an error of 1′ in reading the angle of total reflection

These results are not in good agreement with those obtained by Hammarsten[10] and by Lewinski,[11] who agreed in stating that the percentage of globulins exceeds the percentage of albumins in the serum of the horse I have had occasion to note a similar divergence between my results and those of Hammarsten, employing ox serum [12] This divergence is the more surprising since, as we shall see, the agreement between my results and those of Hammarsten and other observers employing the serum of fully fed (*i e*, "normal") rabbits is highly satisfactory In view of the marked influence of fasting upon the relative proportion of the globulins and albumins in blood sera, which we will discuss in sections C and D of this article, I am inclined to attribute these divergencies to the fact that the horse and oxen from which I obtained my sera were in a fasting condition,[13] while those employed by Hammarsten and Lewinski were not improbably fully fed animals I am confirmed in this belief by the fact that in one instance Hammarsten obtained ox serum containing a greater percentage of albumins than of globulins

[10] O Hammarsten *Arch f d ges Physiol*, xvii, p 413, 1878

[11] J Lewinski *ibid*, c, p 611, 1903

[12] T Brailsford Robertson *loc cit*

[13] The oxen killed at local slaughter-houses are invariably fasting and have usually travelled long distances by railway without food

C Results obtained with rabbit serum

I have carried out a number of analyses of the blood serum of rabbits The animals employed were all of medium size (2000 grams or thereabouts) and were bled directly from the carotid artery into a small Erlenmeyer flask containing glass beads, which were agitated until the blood was defibrinated The blood was then immediately centrifugalized, and the clear serum pipetted off and analyzed

For my earlier analyses I employed serum obtained from "normal" animals, animals, that is, taken directly from the animal house and fed at an undetermined time prior to the bleeding The following were the results obtained

TABLE 3 *Normal rabbits*

RABBIT NUMBER	INSOLUBLE GLOBULINS	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
	per cent	*per cent*	*per cent*	*per cent*
1 (Gray)		1 7 ± 0 15	4 9 ± 0 2	6 6 ± 0 2
2 (Yellow)	0 35 ± 0 04	2 0 ± 0 15	4 5 ± 0 2	6 5 ± 0 2
3 (Yellow)		2 1 ± 0 15	5 0 ± 0 2	7 1 ± 0 2
Average	0 35 ± 0 04	1 9 ± 0 15	4 8 ± 0 2	6 7 ± 0 2

Expressing each of the above mentioned proteins or groups of proteins in terms of the percentage of the total proteins which they represent, the above results for "normal" rabbit serum may be summarized as follows

"Insoluble" globulin	5 2	(± 0 4)
Total globulins	$28 \begin{Bmatrix} 31 \\ 26 \end{Bmatrix}$	(± 2)
Total albumins	$72 \begin{Bmatrix} 74 \\ 69 \end{Bmatrix}$	(± 2)

The first figure opposite each group represents the average percentage, the upper figures immediately following, the highest percentage observed in any individual, the lower figure the lowest percentage observed in any individual, and the figure in brackets, the plus or minus error in the estimation of these percentages which would be brought about by an error of 1′ in reading the angle of total reflection

From these results, it would appear that the variations in the relative proportions of the three groups of proteins considered

in the three different individuals employed are not greater than the variations which might arise from experimental errors in the determinations The above results may be compared with the following previous estimates of the total globulins, albumins and total proteins in rabbit serum

O Hammarsten,[14] determinations made upon four animals, globulins precipitated by saturation of the serum with $MgSO_4$

Total globulins	$29 \begin{Bmatrix} 32 \\ 25 \end{Bmatrix}$
Total albumins	$71 \begin{Bmatrix} 75 \\ 68 \end{Bmatrix}$

C Inagaki,[15] determinations made upon thirteen animals, globulins precipitated by half-saturation of the serum with Am_2SO_4

Total globulins	$29 \begin{Bmatrix} 36 \\ 21 \end{Bmatrix}$
Total albumins	$71 \begin{Bmatrix} 79 \\ 64 \end{Bmatrix}$

These data, obtained by precipitation of the globulins and subsequent Kjeldahl determinations of the nitrogen contained in the precipitate and in the coagulated proteins of whole serum are in good accord with the data obtained by the much less laborious refractometric method The individual variations in the ratio of globulin to albumin which are recorded by Hammarsten and Inagaki are, however, large—much too large it would appear to be attributed to experimental errors in the determinations It occurred to me, however, that these variations might in large part be attributable to the presence in the blood of varying quantities of protein absorbed from the intestinal tract, and the possibility suggested itself that the relative proportions of the serum proteins in a fasting animal might be less subject to individual variation and more typical of the animal species under investigation Accordingly, a number of determinations, similar to the above, were carried out, the rabbits employed, however, being animals which had fasted, with free access to water, for a period of five days preceding bleeding The following were the results obtained

[14] O Hammarsten *Arch f d ges Physiol*, xvii, p 459, 1878
[15] C Inagaki *Zeitschr f Biol*, xlix, p 77, 1907

TABLE 4 *Fasting rabbits*

RABBIT NUMBER	INSOLUBLE GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS	TOTAL PROTEINS
	per cent	*per cent*	*per cent*	*per cent*
1 (Gray)		1 2 ± 0 15	6 8 ± 0 2	8 0 ± 0 2
2 (Gray)		1 4 ± 0 15	7 2 ± 0 2	8 6 ± 0 2
3 (Gray)	0 21 ± 0 04	1 3 ± 0 15	6 2 ± 0 2	7 5 ± 0 2
4 (Black)	0 21 ± 0 04	1 2 ± 0 15	6 8 ± 0 2	8 0 ± 0 2
5 (Gray)	0 24 ± 0 04	1 3 ± 0 15	5 6 ± 0 2	6 9 ± 0 2
6 (Gray	0 24 ± 0 04	1 2 ± 0 15	6 2 ± 0 2	7 4 ± 0 2
7 (long haired albino)	0 24 ± 0 04	1 3 ± 0 15	6 0 ± 0 2	7 3 ± 0 2
Average	0 23 ± 0 04	1 3 ± 0 15	6 4 ± 0 2	7 7 ± 0 2

Expressing each of the above mentioned proteins or groups of proteins in terms of the percentage of the total proteins which they represent, these results for fasting rabbit's serum may be summarized as follows

"Insoluble" globulins $3\ 0\left\{\begin{matrix}3\ 5\\2\ 6\end{matrix}\right\}(\pm\ 0\ 4)$

Total globulins $17\left\{\begin{matrix}18\\15\end{matrix}\right\}(\pm\ 2)$

Total albumins $83\left\{\begin{matrix}85\\82\end{matrix}\right\}(\pm\ 2)$

From these results we perceive

(1) That the percentage of total proteins in sera derived from different fasting individuals is highly variable, a fact which is readily understood when we reflect that the animals were allowed free access to water

(2) That the relative proportions of the different proteins in the sera of fasting animals are constant within the limits of the experimental error of the determination

(3) That during starvation the total protein content of the blood serum increases (compare tables 3 and 4)

(4) That during starvation the percentage of globulins in the blood serum diminishes

(5) That, consequently, during starvation the proportion of albumins to globulins undergoes a marked increase

An increase in the total protein content of blood serum during starvation has been observed by Simon[16] in the horse and by Lewinski[17] and Githens[18] in the dog. The period of starvation employed by Simon and Lewinski being from three to six days, while that employed by Githens was from two to three weeks. Salvioli[19] and Burckhardt[20] employing a period of from three to six days' starvation in dogs found no perceptible alteration in the protein content of the serum, while Panum[21] employing a period of thirteen days' starvation in dogs found an actual decrease in the total protein content of the serum.

Having regard to the fact that the methods of determining the protein content of fluids have undergone great improvement since Panum, Salvioli and Burckhardt carried out their estimations, these results are in good accord with my own results cited above. As regards the alteration in the proportion of globulins to albumins consequent upon starvation, however, much divergence of opinion is expressed by different investigators. Thus Salvioli finds no alteration in the proportion of globulins to albumins in the blood serum of dogs during starvation, while Burckhardt, also employing dogs, finds an increase in the "insoluble" globulin (precipitated by dialysis) and a corresponding decrease in the percentage of residual protein. Lewinski, who separates the globulins by saturation with $MgSO_4$, finds an increase both in globulins and in albumins in dog serum during starvation, the increase in globulins being more marked than the increase in albumins. Githens, who separates the globulins by fractional coagulation with NaCl, finds a decrease in the proportion of albumins and a corresponding increase in the proportion of globulins in dog serum during starvation. The concensus of opinion appears to be, therefore, that in dogs fasting leads to an increase in the proportion of globulins to albumins.

[16] Simon. *Physiologische und pathologische Anthropochemie*, Berlin, 1842, p. 235, cited after Panum. *Virchow's Archiv*, xxix, p. 241, 1864.

[17] J. Lewinski. *Arch. f. d. ges. Physiol.*, c, p. 611, 1903.

[18] T. Githens. *Beitr. z. chem. Physiol.*, v, p. 514, 1904, cf. also V. Subbotin. *Zeitschr. f. Biol.*, vii, p. 185, 1871, J. G. Otto. *Maly's Jahresbericht f. Tierchem.*, xvii, p. 134, 1888.

[19] G. Salvioli. *Arch. f. (Anat. u.) Physiol.*, 1881, p. 269.

[20] Burckhardt. *Arch. f. exp. Path. u. Pharm.*, xvi, p. 322, 1883.

[21] P. D. Panum. *loc. cit.*

H T Krieger,[22] however, employing rabbits, obtains the same result as I, namely, a *decrease* in the proportion of globulins to albumins during starvation It appears, therefore, that the direction in which this ratio alters during starvation varies with the species, possibly with the nature of the normal dietary of the animal As we shall see, my results obtained with the sera of normal and of fasting rats confirm this view

D Results obtained with rat serum

Twelve normal (that is, fully fed) adult albino rats were etherized and bled by cutting the throat The blood of all of the animals was caught in the same Erlenmeyer flask containing glass beads and defibrinated by shaking It was then immediately centrifuged, and the clear serum pipetted off and analyzed The following were the results obtained

TABLE 5 *Normal rats*

	per cent
"Insoluble" globulin	0 48 ± 0 04
Total globulin	1 7 ± 0 15
Total albumins	4 8 ± 0 2
Total proteins	6 5 ± 0 2

Expressing the above results, as in previous cases, in terms of percentages of the total proteins, we obtain for normal rat serum

"Insoluble" globulin	7 4 (± 0 4)
Total globulin	26 (± 2)
Total albumins	74 (± 2)

Similar determinations were carried out upon the serum of fasting rats Eighty animals were fasted for thirty-six hours[23] They were bled in batches of forty into two separate Erlenmeyers, the blood being defibrinated with glass beads and then centrifuged as before The following were the results obtained

[22] H T Krieger Inaugural Dissertations Strassburg, 1899, cited after *Maly's Jahresbericht f Tierchem*, 1899, p 14

[23] It is impossible to fast rats much longer than this without inducing pathological symptoms After sixty hours they die of hunger or else, if kept together, commence devouring each other

TABLE 6 *Fasting rats*

	BATCH 1 per cent	BATCH 2 per cent
"Insoluble" globulin	0 55 ± 0 04	0 55 ± 0 04
Total globulins	2 6 ± 0 15	2 7 ± 0 15
Total albumins	4 8 ± 0 2	4 8 ± 0 2
Total proteins	7 4 ± 0 2	7 5 ± 0 2

Whence we may conclude

(1) That in rats, as in rabbits and dogs, starvation leads to an increase in the protein content of the blood serum

(2) That in rats, as in dogs, the effect of starvation upon the ratio of globulin to albumin is contrary to its effect upon this ratio in rabbits, that is to say, the proportion of globulins to albumins *increases*

Expressing the above results, as in previous cases, in terms of percentages of the total proteins, we obtain for fasting rat's serum

"Insoluble" globulin	7 4 (± 0 4)
Total globulins	36 (± 2)
Total albumins	64 (± 2)

II DISCUSSION OF THE RESULTS

Summarizing the results enumerated above, obtained with fasting animals, and comparing them with the results previously obtained with the serum of fasting oxen, we obtain the following table in which the results are expressed as percentages of total protein

TABLE 7 *Fasting animals*

SPECIES	INSOLUBLE GLOBULIN	TOTAL GLOBULINS	TOTAL ALBUMINS
Albino rat	7 4 (±0 4)	36 (± 2)	64 (± 2)
Rabbit	3 0 $\begin{Bmatrix} 3\ 5 \\ 2\ 6 \end{Bmatrix}$ (±0 4)	17 $\begin{Bmatrix} 18 \\ 15 \end{Bmatrix}$ (± 2)	83 $\begin{Bmatrix} 85 \\ 82 \end{Bmatrix}$ (± 2)
Ox	8 9 (±0 4)	36 (± 2)	64 (± 2)
Horse	4 2 (±0 4)	43 (± 2)	57 (± 2)

Assuming that the divergence between my results for horse and ox sera and those of previous observers, alluded to above, is due to the fact that I employed the sera of fasting animals, while others have employed the sera of fully fed animals, we may construct

a similar tabular comparison of the sera of "normal," *i e*, fully fed animals, as follows

TABLE 8 *Normal animals*

SPECIES	INSOLUBLE GLOBULINS	TOTAL GLOBULINS	TOTAL ALBUMINS
Albino rat (Robertson)	7 4 (±0 4)	26 (± 2)	74 (± 2)
Rabbit (Robertson)	5 2 (±0 4)	$28\left\{\begin{matrix}31\\26\end{matrix}\right\}$ (± 2)	$72\left\{\begin{matrix}74\\69\end{matrix}\right\}$ (± 2)
Rabbit (Hammarsten)		$29\left\{\begin{matrix}32\\25\end{matrix}\right\}$	$71\left\{\begin{matrix}75\\68\end{matrix}\right\}$
Rabbit (Inagaki)		$29\left\{\begin{matrix}36\\21\end{matrix}\right\}$	$71\left\{\begin{matrix}79\\64\end{matrix}\right\}$
Ox (Hammarsten*)		$58\left\{\begin{matrix}64\\54\end{matrix}\right\}$	$42\left\{\begin{matrix}46\\36\end{matrix}\right\}$
Horse (Hammarsten†)		$63\left\{\begin{matrix}71\\58\end{matrix}\right\}$	$37\left\{\begin{matrix}42\\29\end{matrix}\right\}$
Horse (Lewinski‡)		$63\left\{\begin{matrix}69\\56\end{matrix}\right\}$	$37\left\{\begin{matrix}44\\31\end{matrix}\right\}$

* Determinations made upon four individuals the fifth alluded to above (Section B) in which the albumins were present in excess of the globulins is omitted

† Determinations made upon ten individuals

‡ Determinations made upon four individuals

A comparison of these tables enables us to draw the following inferences

(1) In the rabbit, ox and horse, which are herbivorous animals, starvation leads to an increase in the proportion of albumins to globulins in serum, while in the rat, which is omnivorous, and (cf above) in the dog, which is carnivorous, starvation leads to an increase in the proportion of globulins to albumins in the serum These facts, although as yet insufficiently extended to afford a safe basis for generalization, suggest rather forcibly the view that the effect of starvation upon the ratio of globulin to albumin in mammalian blood sera is correlated with the nature of the normal dietary and, conversely, that the nature of the normal dietary is determined by the composition of the blood and the needs of the tissues no less than by the character of the alimentary canal If this be indeed the case then the effect of starvation upon the rats employed in my investigations is very surprising in view of the fact that they had been fed for many weeks preceding the analyses upon *a purely vegetable diet*, to wit, crushed barley

The great constancy of the ratio of "insoluble" globulin to total globulin and to total albumins, in different fasting individuals of the same species (cf table 4), and its variation in the different species investigated, indicate that it is a specific character In fully fed animals the individual variations are greater, nevertheless the data enumerated in table 8 indicate that the average value of this ratio is a specific character for fully fed animals also A comparison of tables 7 and 8 would even appear to indicate that phylogenetic relationships are better displayed in the protein composition of the sera of "normal" than in that of the sera of fasting animals, for in table 8 the two rodent sera are clearly seen to be related and differ markedly from the two ungulate sera which also resemble one another In table 7 these relationships are not so clearly displayed As yet, the data at hand are too scanty to enable one to determine in favor of the one or of the other standard of comparison A comparison between the sera of fully fed animals, however, obviously necessitates a much larger number of determinations than a comparison between the sera of fasting animals

I have elsewhere[24] pointed out that we are in possession of many data tending to show that the proteins in blood serum are united to form a complex, the character of which is presumably determined by the relative proportions in which the different proteins enter into it, and I have suggested that the specific character of this complex in different species may possibly underlie the phenomenon of specificity in the immunological sense The fact that starvation alters the proportion in which the various proteins are present in the sera of animals does not conflict with this view, while the high degree of constancy in the relative proportion of the various proteins in the sera of fasting animals lends some degree of support to it

The investigations of Voit and the more modern investigations of Folin,[25] Schreuer[26] and others have taught us to distinguish between *circulating protein*, which is only transitorily resident in the body, and *tissue protein*, which is bound up with the maintenance of life and not readily catabolized We may well suppose

[24] T Brailsford Robertson *Univ of Calif Publ Physiol*, IV, p 25, 1911, *Die physiologische Chemie der Proteine*, Dresden, 1912, pp 126–132

[25] O Folin *Amer Journ of Physiol*, XIII, p 117, 1904

[26] M Schreuer *Arch f d ges Physiol*, CX, p 227, 1905

that protein of the former character, derived from the alimentary canal and in process of being carried to the tissues by the blood stream, does not form an integral part of the specific serum-protein complex to which I have referred In fully fed animals such "circulating protein" would be present in the blood in *maximum* concentration, while in the blood of fasting animals, in accordance with the metabolic "law of minimum," we should expect "circulating" readily catabolized protein to be present in *minimum* concentration

III SUMMARY

The quantities of "insoluble" globulin, total globulin and total albumin contained in the sera of the normal (fully fed) rat and rabbit and of the fasting horse, rat and rabbit have been determined by a refractometric method described in a previous communication During the course of this investigation the following conclusions were reached

(1) The refractometric method of analysis yields results (with the rabbit) which closely agree with those obtained by previous observers, employing other methods of analysis

(2) Horse serum does not contain preformed crystallizable albumin to the extent of more than 40 per cent of the total albumins

(3) The refractometric method cannot be employed to determine more precisely than this the quantity of crystallizable albumins contained in sera

(4) Provided not over 40 per cent of the albumins are of the crystallizable variety, no appreciable error is introduced into the refractometric determinations of the total albumins and total proteins in sera by assuming that all of the albumins present have the refractivity of amorphous serum albumin, to wit, 0 00177 per gram per 100 cc of solvent

(5) The relative proportion in which the above mentioned three groups of proteins are present in the serum of fully fed animals is subject to rather high individual variations, the *average* values are, however, characteristic of the species from which the serum is derived

(6) The percentage of total proteins in sera derived from different fasting individuals (with free access to water) is highly variable

(7) The relative proportions in which the above mentioned three groups of proteins are present in the sera of fasting animals of the same species (rabbits) is constant within the limits of the experimental error of the determination

(8) During starvation, the total protein content of the blood-serum rises

(9) In the rabbit, ox and horse starvation leads to an increase in the proportion of albumins to globulins in the serum, while in the rat and in the dog starvation leads to an increase in the proportion of globulins to albumins in the serum

PUTREFACTION WITH SPECIAL REFERENCE TO THE PROTEUS GROUP

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(Received for publication, October 15, 1912)

The subject of putrefaction has caused much confusion in the minds of different investigators Most of the definitions of the term putrefaction which may be found in text-books, dictionaries, etc, are so vague and in many instances contradictory that little information may be gained from them Some authors regard all bacterial decompositions of organic matter, particularly proteins, carbohydrates and fats, as putrefactive changes Others would limit the action to proteins alone without special reference to the actual nature of the decomposition or to the products Others, again, would restrict the meaning so as to include only those processes of protein disintegration which give rise to foul-smelling products

In previous publications[1] one of us accepted the last-mentioned view, namely, that of Bienstock[2] and numerous other investigators Since then there has been no occasion to depart from that view It may be said that there is no word in the English language which conveys exactly the meaning that the word "Faulniss" does, yet the term "putrefaction" is of practically the same significance It is derived from the Latin "putrere," meaning "to be rotten," and hence may be defined literally as a process of rotting or offensive decay

There is no very serious objection to giving the word putrefaction the more popular meaning which is so generally applied to it, as a matter of convenience, but in its scientific application its meaning should be more specific or restricted Putrefaction

[1] Rettger this *Journal*, ii p 71, 1906, *ibid*, iv, p 45, 1908

[2] Bienstock *Arch f Hyg* xxxvi, p 335, 1899, *ibid*, xxxix, p 390, 1901

may be defined, therefore, as a particular process of protein decomposition which is brought about through the agency of bacteria with the evolution of foul-smelling products which are characteristic of ordinary cadaveric decomposition It should be stated that mercaptan is of particular significance and that indole, skatole and hydrogen sulphide are of less importance

Pasteur was the first to point out that putrefaction is essentially an anaerobic process This view has since been held by many investigators Certain writers have in recent years attempted to overthrow the conception of Pasteur, however Whether the newer contentions are based on sufficient experimental evidence may be regarded by many as an open question

In a previous paper[3] it was claimed that only certain obligate anaërobes are able to initiate and carry on the decomposition of native proteins in the absence of atmospheric oxygen *Bacillus putrificus*, *B edematis maligni* and *B anthracis symptomatici* were mentioned as the best examples *B tetani* does not have a place in this group *B aërogenes capsulatus* (Welch) has only a limited if, in fact, any proteolytic action All of the facultative anaerobes that were tried were found to lack this power At least thirty different organisms, including *Proteus vulgaris*, were subjected to the test The results were in perfect harmony with those which Bienstock[4] obtained in his extensive work on putrefaction

During the past two years we have repeated some of the earlier experiments, and particularly those bearing on the proteus group As a preliminary part of the investigation, we sent personal requests to at least twenty-five different bacteriological laboratories for culture tubes of members of the proteus group of bacilli We were liberally supplied with cultures from many sources, including four strains from the Pasteur Institute of Paris (Professor Metchnikoff's laboratory) Of the tubes received, twelve were labeled *Proteus vulgaris*, three, P *mirabilis*, two, *P zenkeri*, one, *P hominis* and one, *P versicolor* Furthermore, there were tubes bearing the numbers 370, 372, 374 (1) and 374 (2) and four cultures from the Pasteur Institute labeled Proteus a, b, c and d

[3] Rettger this *Journal*, ii, p 71, 1906

[4] Bienstock *loc cit*

All of the strains were put through the most important cultural and microscopic tests in order to satisfy ourselves that we were in each case working with a member of the so-called proteus group and with uncontaminated cultures The putrefaction tests were usually made in the egg-meat medium[5] which has for several years been employed in this laboratory Tubes of blood fibrin suspended in bouillon and in Uschinsky medium were employed also The egg-meat medium was used in preference to others because of the ease with which it is prepared and the satisfactory results which have been obtained with it

All of the tests were made in large test tubes Anaerobiosis was induced by means of the alkali-pyrogallic acid mixture, by the Buchner and the Wright methods As checks, putrefaction tests were made with *B putrificus*, *B edematis maligni* and *B anthracis symptomatici* Positive putrefaction results were obtained with these organisms in every instance, that is, on opening the tubes the characteristic odor of putrefaction was given off and the solid matter (egg white and meat) was rapidly reduced in volume

With the various members of the proteus group the results were entirely different Not one of the twenty-six strains which were used in this investigation gave us the slightest indication that it possessed the ability to initiate and bring about any visible change in the character of the protein material, when grown under anaërobic conditions There was no visible reduction in the volume of the solid matter nor the least odor of putrefaction when perfectly clean rubber stoppers were employed in sealing the tubes In a very few instances a slight odor of putrefaction was perceived, but this was found to be due to stoppers which had been used in positive putrefaction tests On removing the stopper and cotton plug, and after thorough cleansing of the mouth of the tube, the odor disappeared

Tubes were left in the incubator (37 5°C) for at least a week, often for two or three weeks They were also kept at ordinary room temperature for similar periods Before incubation at the higher temperature the tubes were kept comparatively cool (8–10°) for from twenty-four to thirty-six hours in order to

[5] Rettger this *Journal*, ii, p 71, 1906

allow the oxygen to be absorbed before any aerobic bacterial action could take place All of the tests were duplicated and duplicate examinations of each organism were made at least three or four times

Rigid microscopic examinations were always made of the contents of the proteus-inoculated tubes and, whenever there was any question of contamination, cultural tests were made The organisms used in the inoculation were always detected with ease and their numbers indicated that considerable multiplication had taken place The growths were made possible by the presence of non-protein nitrogenous substances and not by the proteins

Besides the anaerobic tests numerous examinations were made under aerobic conditions In so far as actual putrefaction is concerned, the results were again all negative *Proteus zenkeri* has no visible disintegrating action whatever *P mirabilis* likewise has none or, at the most, very little when grown under aërobic conditions On the other hand, *P vulgaris* possesses the ability to attack and to a certain extent decompose the egg-meat proteins and blood fibrin Such action is, however, very slow and is dependent on an ample supply of atmospheric oxygen In such decomposition of protein there are a number of well-known bacterial decomposition products as indole, amines, amino-acids, hydrogen sulphide, etc , but these are not the products of real putrefaction, in the sense in which we use the term, and the odor given off from the *P vulgaris* tubes is not that which is so characteristic of putrefactive changes, as illustrated in the action of *B putrificus*

Many of the common aërobes and facultative anaërobes are known to have definite proteolytic action on egg albumin, serum albumin, etc , when in contact with free oxygen This is demonstrated easily on ordinary blood serum or egg white when they have been coagulated by heat, and we need not seek any further for organisms than *B subtilis*, *B prodigiosus*, *B pyocyaneus*, etc Furthermore, indole and hydrogen sulphide are very common protein decomposition products, and are not indicators of real putrefaction The results are quite different, however, when oxygen is excluded, as we have so frequently observed

We are forced to disagree, therefore, with Tissier and Martelly,[6] who claim that *Proteus vulgaris* exerts a putrefactive influence on proteins When inoculated by them into Uschinsky-Fraenkel medium containing blood fibrin the organism caused the medium to become clouded in twenty-four hours and, according to them, a fetid odor was given off in the course of two days The fibrin was attacked At the end of fifteen days the action seemed to be arrested On analysis they found indole, phenol, amines, leucine, acetic, formic, butyric and valerianic acids and hydrogen sulphide No mention is made of mercaptan or the aromatic oxy-acids In its action on proteoses the organism produced the same products

They succeeded in isolating an enzyme which acts in neutral, alkaline or slightly acid solution, but which is much less active than that of the anaerobes studied They found *P zenkeri* associated with *P vulgaris* in spontaneous putrefaction mixtures, but were unable to demonstrate the presence of a proteolytic enzyme in cultures of *P zenkeri* This organism was unable to attack native proteins

We have no comments to make on the question as to whether the action of P *vulgaris* on the blood fibrin, as observed by Tissier and Martelly, was one of real putrefaction, in the sense that we use the term Their observations were made on a very limited number of strains of *P vulgaris*, if indeed more than one On the other hand, at least eighteen different strains of the organism were tested by us, and in every instance with negative results

Basing his conclusions largely on the observations of Tissier and Martelly, Metchnikoff[7] holds to the view that putrefaction may be induced by certain aerobes

The putrefactive anaerobes and certain members of the proteus group are frequently associated with each other While they appear to be present but rarely in the normal intestines of man, they are found side by side in the spontaneous decomposition of protein-containing substances, like meat, humus, etc The two groups assume different rôles in the processes of disintegration of protein and in putrefaction Together with other organisms which are essentially aërobic, the *P vulgaris* exerts an early dis-

[6] Tissier and Martelly *Ann de l'Inst Pasteur*, xvi, p 865, 1902
[7] Metchnikoff *ibid*, xxii, p 928, 1908

None of these substances with the possible exception of the imidazole nucleus have been demonstrated to be destroyed when introduced into the organism Dakin and Wakeman[1] have shown by perfusion experiments with the liver that some slight decomposition of histidine, which contains the imidazole nucleus, may take place with the formation of acetoacetic acid, but they conclude that the effect is too slight to formulate any promising hypothesis for the catabolism of histidine Feeding experiments with histidine[2] leave the fate of this substance in the organism in doubt The failure of these related compounds to experience disintegration in metabolism renders the behavior of hydantoin, a compound simpler than any of the others, of particular interest

From another viewpoint, the behavior of hydantoin seems worthy of study Lusini[3] working with alloxan and alloxantin reached the conclusion that the grouping $\begin{array}{l}NH-\\ \;|\\ C=O\\ \;|\\ NH-\end{array}$ functions to stimulate and then inhibit nerve centers It is, according to Lusini, the ketone-like group $>CO$ which has the stimulating property and an abundance of these groups increases the toxicity More recently Kleiner[4] was unable to confirm Lusini's conclusions since barbituric acid, which Kleiner studied and which is non-toxic, contains the ketone group and differs little from the toxic substance alloxan Inasmuch as hydantoin also contains the alleged toxic group, $\begin{array}{l}NH-\\ \;|\\ CO\\ \;|\\ NH-\end{array}$, the question of its toxicity is of interest

The present paper deals with the behavior of hydantoin and

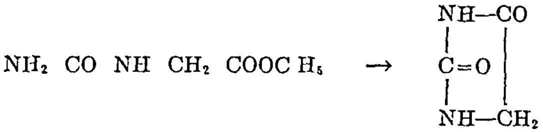
[1] Dakin and Wakeman this *Journal*, x, p 499, 1912

[2] Abderhalden and Einbeck *Zeitschr f physiol Chem*, lxii, pp 322-32, 1909, Abderhalden, Einbeck and Schmid *ibid*, lxviii, pp 395-99, 1910, Kowalevsky *Biochem Zeitschr*, xxiii, pp 1-4, 1910

[3] Lusini *Ann di chim e di farmacol*, xxi, pp 145-60, 241-57, xxii, pp 341-51, 385-94, 1895, *Chem Centralbl*, i, p 1074, ii, p 838, 1895

[4] Kleiner this *Journal*, xi, pp 443-70, 1912

the ethyl ester of hydantoic acid, introduced in different ways into the organism of various species The hydantoic acid ester was prepared from glycocoll ester hydrochloride and potassium cyanate, according to the method of Harries and Weiss[5] On evaporating the ester to dryness with concentrated hydrochloric acid, it is converted to the hydantoin The latter is then purified by recrystallization from absolute alcohol



Ethyl ester of hydantoic acid Hydantoin

Analysis (Kjeldahl nitrogen determination) of the hydantoin prepared gave the following result

	Calculated for $C_3H_4N_2O_2$	Found
N	28 00 per cent	27 88 per cent

For the identification of the hydantoin in the urine, use was made of the insoluble benzalhydantoin The urine was acidified and evaporated to small volume on a water bath, decolorized with animal charcoal and evaporated to dryness The product was then condensed with benzaldehyde in the presence of glacial acetic acid, acetic anhydride and dried sodium acetate, as described by Wheeler and Hoffman[6] For the identification of the ester, the urine was evaporated to dryness with concentrated hydrochloric acid to convert the ester into hydantoin, and the benzal derivative was prepared as before

The analytical procedures included the Kjeldahl-Gunning method for nitrogen and Folin's methods for urea and creatinine Blank experiments with hydantoin showed that this substance is not attacked by Folin's urea method

In the experiments with rabbits the bladder was emptied by pressure at the same hour daily The substances, when fed, were dissolved in water and introduced through a gastric sound In the experiments with dogs the animals were catheterized at reg-

[5] Harries and Weiss *Ber d deutsch chem Gesellsch*, xxxiii, p 3418, 1900

[6] Wheeler and Hoffman *Amer Chem Journ*, xlv, p 368, 1911

ular twenty-four-hour intervals Here the substances fed were mixed with the food

EXPERIMENTS WITH HYDANTOIN

Rabbit I Diet, 300 grams of carrots daily This was completely consumed except on the day of the hydantoin administration when the animal ate only 270 grams No toxic effects of any sort were noted The protocol follows

Rabbit, weight, 1 8 kgms

Day	Volume	Specific gravity	Total N	Urea + NH_3 N	$\frac{\text{Urea} + NH_3 \text{ N}}{\text{Total N}}$	N not urea + NH_3 N	Remarks
	cc		*grams*	*gram*	*per cent*	*gram*	
1	280	1 012	0 876	0 768	87 6	0 108	
2	295	1 012	0 918	0 750	80 1	0 168	
3	245	1 015	0 900	0 756	84 0	0 144	1 5 gm hydantoin
4	145	1 026	**1 308**	0 738	**56 4**	**0 570**	= 0 42 gram N, in
5	220	1 016	0 696	0 600	86 4	0 096	traperitoneally
6	205	1 024	0 690	0 630	90 1	0 060	

About 0 3 gram of benzalhydantoin, after purification by recrystallization from alcohol, was obtained from the urine of the fourth day The benzalhydantoin isolated melted at 217° and when mixed with a pure synthetic sample did not alter the melting point of the latter

No increase in the urea + ammonia nitrogen on the day of the injection was observed, although the increase in the elimination of total nitrogen excreted accounted for all the nitrogen administered as hydantoin This fact, together with the identification of hydantoin in the urine, indicates that hydantoin is unaltered in its passage through the body

Another experiment with the same animal a few days later, in which the same amount of hydantoin was administered *per os*, gave similar results

Rabbit II Diet, 300 grams of carrots On the day on which

the hydantoin was fed, the animal consumed the full daily ration No toxic symptoms of any sort were noted The protocol follows

Rabbit, weight, 1 64 kgms

DAY	VOLUME	SPECIFIC GRAVITY	TOTAL N	UREA + NH₃ N	(UREA + NH₃-N)/TOTAL N	N NOT UREA + NH₃ N	REMARKS
	cc		gram	gram	per cent	gram	
1	275	1 015	0 900	0 786	87 3	0 114	
2	240	1 018	0 708	0 642	90 6	0 066	
3	150	1 016	0 624	0 558	89 4	0 066	1 5 gm hydantoin
4	205	1 021	**0 918**	0 576	**62 7**	**0 342**	= 0 42 gram N,
5	280	1 019	0 726	0 570	79 1	**0 156**	*per os*
6	160	1 020	0 600	0 516	86 0	0 084	
7	160	1 025	0 570	0 474	83 1	0 096	

From the urine of the experimental day a small amount of benzalhydantoin melting at 218° was obtained

Dog A female was fed on a constant daily diet of 200 grams of lean meat, 50 grams of lard, 30 grams of sugar, 5 grams of bone ash, 2 grams of salt and 250 cc of water with a total nitrogen content of 6 84 grams The hydantoin was dissolved in the water of the diet The animal ate eagerly on the experimental day as at other times

Dog A, weight, 12 4 kgms

DAY	VOLUME	SPECIFIC GRAVITY	TOTAL N	UREA + NH₃ N	(UREA + NH₃ N)/TOTAL N	N NOT UREA + NH₃ N	CREATININE	REMARKS
	cc		grams	grams	per cent	grams	gram	
1	160	1 055	8 45	7 63	89 8	0 82	0 279	
2	180	1 042	6 69	6 24	93 3	0 45	0 294	
3	300	1 025	6 38	5 80	90 9	0 58	0 294	2 5 gm hydantoin =
4	300	1 030	**6 87**	5 67	**82 6**	**1 20**	0 298	0 7 gm N
5	320	1 024	5 99	5 39	90 0	0 60	0 306	with food
6	350	1 023	6 12	5 44	88 8	0 68	0 284	
7	300	1 028	6 37	5 76	90 4	0 61	0 297	

From the urine of the experimental day 3.3 grams of benzalhydantoin were obtained corresponding to 1.7 grams of hydantoin (= 0.476 gram N) in the day's urine. This was purified by solution in potassium hydroxide and reprecipitation with acid.

Cat. A cat weighing approximately 4 kgms. received 3 grams of hydantoin mixed with raw meat. The urine of the next twenty-four hours was collected and examined for the presence of hydantoin as above. The benzalhydantoin obtained melted at 214° and after purification weighed 1.7 grams.

A nitrogen determination (Kjeldahl) on the mixed products obtained in this and the three preceding experiments gave the following results.

	Calculated for $C_{10}H_8N_2O_2$	Found
N	14.92 per cent	14.59 per cent

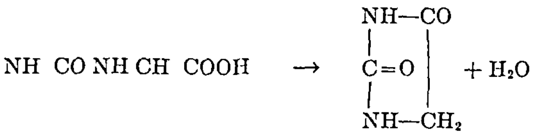
In all our experiments the recovery of the administered hydantoin from the urine leaves no doubt as to its absorption. The increase in the total nitrogen of the urine on the experimental day also points to the same conclusion. In no case was the urea + ammonia nitrogen output increased, the difference between the total and urea + ammonia nitrogen nearly always approaching the value of the nitrogen administered as hydantoin. In the rabbit II, there was observed a slight lag, part of the hydantoin probably being eliminated on the day after the administration. No effect on the creatinine elimination was observed in the dog.

Hydantoin appears to be without influence on nitrogenous metabolism and is not destroyed or changed by the organism. No toxicity attributable to the group $\begin{array}{c}\mathrm{NH—}\\ | \\ \mathrm{C=O}\\ | \\ \mathrm{NH—}\end{array}$ as alleged by Lusini was observed.

EXPERIMENTS WITH ETHYL HYDANTOATE

In order to ascertain whether the inability of the organism to break down hydantoin was due to the cyclic structure, experiments were conducted with the ethyl ester of hydantoic acid. This acid is converted to hydantoin with the loss of a molecule of water

and bears the same relation to hydantoin that creatine bears to creatinine



Hydantoic acid Hydantoin

Hydantoic acid may also be considered as a uramino acid, uraminoacetic acid Koehne,[7] working with the ethyl ester of the homologous uraminoformic or allophanic acid, found that it disappeared in the body

Rabbit 1 Diet, 300 grams of carrots daily On the experimental day the animal showed no unusual symptoms and resumed eating immediately after the feeding of the ester The protocol follows

Rabbit, weight, 1 7 kgms

DAY	VOLUME	SPECIFIC GRAVITY	CREATININE	TOTAL N	UREA + NH_3 N	UREA + NH_3-N / TOTAL N	N NOT UREA + NH_3 N	REMARKS
	cc		*gram*	*gram*	*gram*	*per cent*	*gram*	
1	220	1 011	0 055	0 810	0 729	90 0	0 091	
2	220	1 012	0 056	0 443	0 347	78 3	0 096	2 gm hydantoic acid ester = 0 38 gram N, *per os*
3	200	1 013	0 047	0 443	0 396	89 8	0 047	
4	295	1 017	0 071	**0 845**	0 420	**49 6**	**0 425**	
5	220	1 016	0 063	0 458	0 347	73 5	0 111	
6	270	1 014	0 061	0 414	0 369	89 1	0 045	

From the urine of the experimental day, benzalhydantoin was prepared in the usual manner An amount equivalent to 0 67 gram of the ester (= 0 13 gram N) was obtained The benzalhydantoin melted at 218° and did not change the melting point of the pure synthetic substance when mixed with it A nitrogen (Kjeldahl) determination gave the following results

	Calculated for $C_{10}H_8N_2O_2$	Found
N	14 92 per cent	14 67 per cent

[7] Koehne Inaugural Dissertation, Rostock, 1894, p 17

Rabbit 2 Diet, 300 grams of carrots daily The animal appeared normal on the day of the injection and ate as usual The protocol follows

Rabbit, weight, 1 5 kgms

DAY	VOLUME	SPECIFIC GRAVITY	TOTAL N	CREATININE	REMARKS
	cc		*grams*	*gram*	
1	95	1 026	1 172	0 103	
2	180	1 020	0 702	0 070	
3	220	1 013	0 611	0 078	
4	210	1 013	**0 878**	0 081	1 5 grams hydantoic acid ester = 0 286 gram N, subcutaneously
5	240	1 012	0 513	0 084	
6	240	1 012	0 374	0 076	
7	230	1 016	0 444	0 073	

From the urine of the experimental day a small amount of benzalhydantoin was prepared which melted at 217° and did not affect the melting point of the pure synthetic substance

Rabbit 3 Diet, 300 grams of carrots and 30 grams of oats daily On the day of the injection only 270 grams of carrots were eaten No abnormal symptoms were noted

Rabbit, weight, 2 34 kgms

DAY	VOLUME	SPECIFIC GRAVITY	TOTAL N	UREA + NH_3 N	$\frac{\text{UREA} + NH_3 \text{ N}}{\text{TOTAL N}}$	N NOT UREA + NH_3 N	CREATININE	REMARKS
	cc		*grams*	*grams*	*per cent*	*gram*	*gram*	
1	265	1 010	1 20	1 020	85 0	0 180	0 122	
2	220	1 013	0 855	0 716	82 9	0 139	0 092	
3	220	1 012	1 018	0 840	83 3	0 180	0 110	
4	260	1 015	**1 476**	1 035	**70 3**	**0 441**	0 145	2 gm hydantoic acid ester = 0 38 grams N intraperitoneally
5	185	1 015	0 996	0 852	85 5	0 144	0 123	
6	160	1 015	0 990	0 900	90 9	0 090	0 135	
7	125	1 018	0 945	0 810	85 7	0 135	0 081	

From the urine of the experimental day benzalhydantoin was prepared and purified by solution in potassium hydroxide and reprecipitation with acid It weighed 0 75 gram = 0 58 gram ester

in the day's urine A nitrogen (Kjeldahl) determination gave the following results

	Calculated for $C_{10}H_8N_2O_2$	Found
N	14 92 per cent	14 88 per cent

Dog A female received a standard daily diet (see experiments on hydantoin) The food was eaten as usual on the experimental day

Dog A, weight, 12 6 kgms

Day	Volume	Specific gravity	Total N	Urea + NH_3 N	$\frac{\text{Urea} + NH_3\text{-N}}{\text{Total N}}$	N not urea + NH_3 N	Creatinine	Remarks
	cc		grams	grams	per cent	grams	gram	
1	175	1 040	6 61	6 03	91 2	0 58	0 349	
2	350	1 026	6 60	6 03	91 3	0 57	0 335	3 gm hydantoic acid ester = 0 67 gm N in food
3	380	1 029	6 59	5 93	90 0	0 66	0 324	
4	300	1 028	**7 34**	6 25	**85 1**	**1 09**	0 326	
5	290	1 033	6 25	5 67	90 8	0 58	0 335	2 gm ester = 0 38 gm N in food See note in the text
6	315	1 026	6 53	5 92	90 7	0 61	0 324	
7	290	1 034	**6 44**	5 55	**86 1**	**0 89**	0 332	

On the seventh day the animal received a second dose of 2 grams of the ester, but refused to eat all the food The animal was stuffed, and some material was lost Hence the urine data on that day cannot be compared with those of the previous days Taken as an isolated experiment, however, the results of the seventh day show the important point that the urea + ammonia nitrogen does not maintain its fairly uniform proportion as on other days but is decreased, indicating excretion of nitrogen in some form other than urea

Small amounts of benzalhydantoin were prepared from the urine of the experimental days The material was darker than that obtained in previous experiments and melted at 210–212° A nitrogen (Kjeldahl) determination gave the following results

	Calculated for $C_{10}H_8N_2O_2$	Found
N	14 92 per cent	14 41 per cent

These experiments all indicate that in the dog and the rabbit the hydantoic acid ester is not affected by the metabolic processes of the body No marked increase of urea and ammonia nitrogen was observed A hydantoin derivative was always isolated from the urine, indicating the presence of the unaltered substance administered The creatinine output of the urine was not affected The very slight increase observed in the experiments, especially with rabbit 2, may be accounted for by the fact that the ester itself gives a slight color with Jaffé's picric acid test as it is applied in the colorimeter

SUMMARY

1 After administration of hydantoin, the compound can be recovered from the urine in the form of an insoluble benzalhydantoin This method is not quantitative, but serves to identify the material

2 No toxic effects were observed to follow the administration of hydantoin This is in opposition to Lusini's theory of the toxicity of

$$\begin{array}{c} \mathrm{NH}— \\ | \\ \mathrm{C} = \mathrm{O} \\ | \\ \mathrm{NH}— \end{array}$$

groups

3 Hydantoic acid, of which hydantoin is the cyclic anhydride, is not destroyed in metabolism when it is administered as the ethyl ester, but can likewise be recovered by forming the insoluble benzalhydantoin

4 The hydantoin nucleus is not destroyed in the organism of the cat, rabbit or dog

These studies were undertaken at the suggestion of Professor Lafayette B Mendel We desire to acknowledge our indebtedness to Professor Treat B Johnson for aid in the synthesis of the compounds employed

THE RACEMIZATION OF PROTEINS AND THEIR DERIVATIVES RESULTING FROM TAUTOMERIC CHANGE

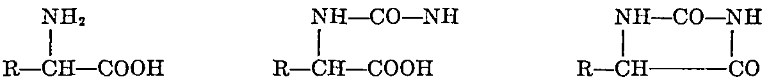
PART I

By H D DAKIN

(From the Herter Laboratory, 819 Madison Avenue, New York)

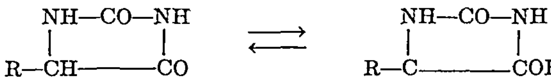
(Received for publication October 21, 1912)

The writer has shown[1] that alkali salts derived from optically active hydantoins (III), prepared from active α-amino-acids (I), undergo spontaneous racemization at room temperature, while the uramido acids (II), intermediate products in the preparation of the hydantoins, like the amino-acids themselves undergo no such change

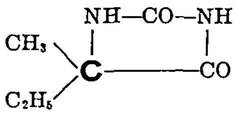


I II III

The racemization of the hydantoin salts was ascribed to tautomeric change of the keto-enol type, represented as follows



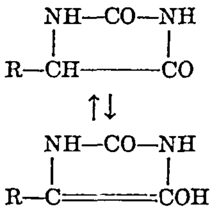
The formation of salts of the enol type, even to a small extent, would necessarily be followed by racemization since the asymmetry of the α-carbon atom is abolished The view that racemization was due to the formation of salts of the enol type was further confirmed by the observation that *d*-methylethylhydantoin,

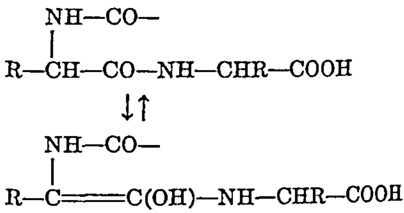


[1] *Amer Chem Journ* , xliv, p 48, 1910

a substance in which no labile hydrogen atom is present, retained its optical activity indefinitely under conditions which resulted in complete racemization in the case of hydantoin salts capable of keto-enol isomerism

A consideration of the changes involved in the racemization of these amino-acid derivatives led to the belief that similar transformations would be found to occur in the proteins and polypeptides [2] For while salts of free amino-acids cannot exhibit keto-enol isomerism, the opposite may be true of certain of the groups in peptide structures The following formulae comparing the hydantoin and peptide groupings, make this clear


Hydantoin


Peptide complex

A consideration of the above formulae would indicate that, of the two amino-acid groups present in the above peptide complex, only the one containing the —CH—CO— group could exhibit keto-enol tautomerism and hence racemization, while the terminal amino-acid group containing a free carboxyl group would be unchanged

This type of change apparently corresponds closely to what takes place when a protein is digested at low temperature with dilute alkali Kossel and Weiss have shown that proteins as widely different as gelatin and clupeine show a rapid decline in optical activity when digested at low temperatures with dilute alkali, but in no case is optical activity completely abolished Kossel and Weiss further show that on hydrolyzing the racemized protamines inactive arginine was obtained, while gelatin gave a variety of products from which on hydrolysis inactive histidine, arginine, ornithine and some inactive lysine were obtained [3]

[2] The racemization changes described in this paper are all effected at low temperatures and have nothing in common with the racemization of amino-acids, etc , at high temperatures when heated with caustic alkalies

[3] *Zeitschr f physiol Chem* , lix, p 492, 1909, lx, p 311, 1909, lxviii, p 165, 1910

Kossel and Weiss regard the phenomenon essentially as one of protein racemization, somewhat complicated by simultaneous hydrolysis due to the action of the alkali Moreover, they came to the conclusion that certain amino-acid groups, *e g*, arginine, undergo racemization much more readily when intramolecularly bound than when free It will be noticed that these conclusions harmonize excellently with the writer's hypothesis and, with Professor Kossel's permission, the writer is attempting to obtain further insight into the mechanism of the action of alkali on proteins

Preliminary experiments upon the action of dilute alkali on gelatin show that certain of the amino-acid groups are readily and completely racemized, while others retain the optical activity unimpaired, while some appear to occupy an intermediate position

The optical properties of some of the amino-acids obtained by hydrolyzing the products resulting from the action of caustic soda upon gelatin, are recorded below

Four hundred gram portions of moist gelatin were dissolved in 4 liters of water and then 200 cc of 33 per cent caustic soda added The initial rotation was approximately $-13.5°$ and sank to $-6.28°$ after twenty-four hours, -3.39 after four days and finally became practically constant at $-2.32°$ after fifteen days The mixture containing ammonia, free amino-acids, peptones and protein was completely hydrolyzed with acids and the mono-amino-acids examined by Fischer's ester method, while the bases were isolated by the methods of Kossel and Kutscher

Glycine was separated as the ethyl ester hydrochloride The glycine was, of course, inactive Yield, about 17 5 per cent

Alanine was obtained in the form of mixtures containing from 25 to 60 per cent of the active form, the balance being inactive Although some racemization takes place during the separation of the alanine, the proportion of inactive substance is rather high and probably indicates some racemization prior to hydrolysis

Leucine Large quantities (about 1 5 per cent) of pure inactive leucine were readily obtained on recrystallizing the "leucine fraction"

ANALYSIS 0 1458 gram gave 0 2950 gram CO_2 and 0 1327 gram H_2O
0 1937 gram gave NH_3 = 0 0209 gram N

	Found	Calculated for $C_6H_{11}O_2N$
C	55 2	55 0
H	10 1	9 9
N	10 8	10 7

All of the leucine that was obtained in a satisfactorily pure state was optically inactive in both aqueous and hydrochloric acid solutions

A portion of the inactive leucine was converted into α-uramido-isobutylacetic acid by means of potassium cyanate On recrystallization from boiling water the substance was obtained in the form of platelets, m p , 203°–204°

The mother liquors from the leucine fractions contained some alanine and were feebly laevorotatory in aqueous solution and dextrorotatory in hydrochloric acid Other amino-acids were apparently present

Proline A yield of proline was obtained about one-half larger than that recorded by Fischer, Plimmer and Levene Its rotation in aqueous solution indicated that it contained about 21 per cent of the laevo variety Since proline is largely racemized in the process of isolation, it is probable that little or no racemization occurred before hydrolysis

The proline was characterized by conversion into the copper salt and into the phenyl-hydantoin

Aspartic acid The aspartic acid isolated in the usual way was completely inactive The yield was 0 6 per cent No indications were obtained of any active aspartic acid

Glutamic acid was separated as hydrochloride Its rotation indicated that no racemization had occurred

ROTATION $\alpha = +3\ 92°$, $l = 2$ dm , $c = 6\ 55$

$$[\alpha]_D^{20} = +29\ 9°$$

Phenylalanine was separated as hydrochloride A 2 per cent solution was completely inactive A yield of slightly over 1 per cent was obtained, or more than twice that previously recorded

ANALYSIS 0 1600 gram gave 0 3845 gram CO_2 and 0 0986 gram H_2O

	Found	Calculated for $C_9H_{11}O_2N$
C	65 5	65 5
H	6 8	6 7

Histidine was isolated by means of the phosphotungstate, silver compound and picrolonate A 3 5 per cent solution of the base dissolved in hydrochloric acid showed a doubtful rotation of −0 08° in a 1 dm tube Racemization was evidently practically complete

Arginine was separated according to Kossel's methods and was inactive The picrolonate melted at 241°–242° and contained 25 4 per cent N (Theory = 25 6)

Lysine and ornithine were precipitated as phosphotungstates after the removal of histidine and arginine The bases were converted into picrates and recrystallized from hot water The less soluble lysine picrate was readily obtained and on decomposition with hydrochloric acid, gave dextrorotatory lysine dihydrochloride

ROTATION $\alpha = +2\ 60°, l = 2$ dm , $c = 9\ 5$

$$[\alpha]_D^{20°} = +13\ 7°$$

Discussion of results The foregoing experiments show that gelatin, when digested with dilute alkali and subsequently hydrolyzed with acids, yields inactive leucine, aspartic acid, arginine, histidine and phenylalanine, while proline, glutamic acid and lysine are obtained in the optically active forms together with part of the alanine It is certainly a striking fact that substances so closely allied as aspartic and glutamic acids or lysine and arginine should exhibit such diametrically opposite behavior

The interpretation of these results is necessarily difficult But in view of the probability of racemization being due to keto-enol, tautomeric change taking place in alkaline solution, and since such racemization can only occur in amino-acid groups in which the carboxyl group attached to the asymmetric carbon atom is in union with other groups (see p 358), the inference appears probable that none of the carboxyl groups in leucine, aspartic acid, arginine, histidine or phenylalanine are free in gelatin On the other hand, since glutamic acid and lysine and possibly alanine are obtained in

active forms, it is possible that some of their carboxyl groups may be free or, in other words, that they *may* occupy terminal positions in the peptide chains On the other hand, it is possible that these amino-acids are rapidly liberated in the free state by the hydrolytic action of the alkali and so escape racemization Further work will be necessary to decide this point

Since simple dipeptides containing a free amino group

$$\mathrm{R\ \underset{\displaystyle NH_2}{\underset{|}{CH}}\text{—}CO\text{—}NH\ CHR\ COOH}$$

or peptides such as prolylglycine, apparently do not undergo ready racemization with dilute alkali it appears likely that the conditions necessary for racemization of an amino-acid group require the attachment of other groups to both amino and carboxyl radicle

In order to fully elucidate the mechanism of the changes involved in the action of dilute alkali on proteins, much additional work is needed and it will be necessary to make experiments with peptides of known structure It would appear that the study may be of service in throwing some light on the relative positions of some of the groups in protein and peptide complexes and, in addition, may furnish information as to the structure of protein salts The investigation is being continued

A NEW METHOD FOR THE (COLORIMETRIC) DETERMINATION OF URIC ACID IN URINE

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(Received for publication, October 26, 1912)

In a previous number of this *Journal*[1] we published a preliminary paper on the color produced by the action of phosphotungstic acid on uric acid and certain other substances and we outlined a method for the determination of uric acid by means of that color reaction Inasmuch as the ordinary method for preparing phosphotungstic acid solutions for this reaction (boiling 20 parts of sodium tungstate with 10 parts of 20 per cent phosphoric acid) gave reagents which had little or no action on uric acid solutions, we began to suspect that the active ingredient was some impurity, variable amounts of which might be present in the sodium tungstate The same impurity might have been present in molybdenum compounds since phosphomolybdic acid also gave variable and not very strong color reactions with uric acid A more systematic investigation[2] soon showed that the amount of active material contained in the phosphotungstic and phosphomolybdic acid solutions was determined by the conditions under which the solutions were prepared and was particularly dependent upon the proportion of phosphoric acid used It was further found that reagents containing phosphomolybdic acid reacted with all phenols as well as with uric acid while the phosphotungstic acid reagents gave the blue color only with polyphenols and with uric acid This point of difference is of considerable importance in respect to a colorimetric determination of uric acid in urine By using the phosphotungstic acid reagent the phenol reaction is in a large measure eliminated Our "uric acid reagent" accordingly is a

[1] This *Journal*, xi, p 265, 1912

[2] Folin and Denis this *Journal*, xii, p 239, 1912

phosphotungstic acid prepared by boiling 100 grams of sodium tungstate with 80 cc of 85 per cent phosphoric acid and 750 cc of water for a couple of hours and then diluting to 1 liter

Since the publication of our first paper E Riegler has published a paper in the *Zeitschrift für analytische Chemie*[3] in which he describes a colorimetric method for the determination of uric acid based on the use of phosphomolybdic acid Although Riegler's new method is quite different from ours, it would seem best to specifically call attention here to the fact that our first paper on the subject was published in the April number of this *Journal* and a second paper which appeared in the August number was received by the Editor of the *Journal* on June 29 Riegler's paper appeared in the seventh and eighth double number of the above mentioned Zeitschrift which was evidently published some time in June and did not reach us until July Riegler evidently has not made any particular study of the color reaction involved or of the conditions under which the maximum color can be obtained He uses phosphomolybdic acid for producing the blue color, and the amount of color which he obtains from a given quantity of uric acid is evidently not nearly as deep as the color which we secure, since his standard color is that given by 1 mgm of uric acid when diluted to 10 cc, while ours is the same amount of uric acid diluted to 100 cc The color which we obtain with 1 mgm of uric acid by means of our reagent would be too dark to transmit any light if diluted to only 10 cc Yet unlike Riegler's reagent, ours gives no color with simple phenols or with protein materials These advantages are of course the result of our having prepared phosphotungstic acid reagents specifically with reference to their chromophoric value, while Riegler uses phosphomolybdic acid prepared for ordinary precipitating purposes

In our earlier work we precipitated the uric acid in urine by a modification of Salkowski's silver method (using silver sulphate instead of nitrate because nitrates interfere with the color reaction) The precipitation, washing, etc, was all done in centrifugal tubes and the final blue solutions were then rinsed into volumetric flasks and made up to volume before reading off the color

After we had obtained a reagent which did not react with protein materials or other monophenol derivatives we hoped to be able to make the color directly on the urine without any preliminary treatment whatever The uric acid values so obtained continue however to be high as compared with the uric acid obtained by the older methods,[4] and we were forced to recognize the fact

[3] *Zeitschr f anal Chem*, li, p 466, 1912

[4] For exact figures indicating the errors which would come in because of these polyphenol derivatives, see column 4, p 369

pointed out long ago by Baumann that urines contain di- and polyphenol acids in addition to the more abundant monophenol derivatives Having obtained a clear idea as to the probable nature of the substances which were responsible for the extra color produced by the uric acid reagent in urine, we have now worked out a new method for the determination of uric acid which is materially simpler than the method outlined in our first paper

This method is described in some detail below

From 2 to 5 cc of urine (the amount depending on the specific gravity) are measured into a 100 cc beaker and after adding a drop of saturated oxalic acid solution the whole is evaporated to dryness on the water bath or over a hot iron plate or even over a free flame This evaporation takes only a few minutes, but it should be continued until the upper parts of the beaker are dry To the dry, cool residue are added 10–15 cc of a mixture consisting of 2 parts of pure, dry ether (*i e*, ether distilled over sodium) and 1 part of pure methyl alcohol After standing for about five minutes the solution is removed by careful decantation and another 10 cc of the alcohol-ether mixture are added to the residue, allowed to settle and decanted The alcohol-ether dissolves out the phenol acids and leaves the uric acid

With regard to this treatment of the evaporated urine residue for the removal of other substances than uric acid which give a blue color with the reagent, it should be said that two very slight sources of error are inherent in the process In the presence of considerable solid residues, soluble phenol acids, as, for example, tannic acid, are not to be perfectly removed by the treatment outlined above Nor is uric acid absolutely insoluble in the organic solvent under the conditions of the experiment These two sources of error are however very slight indeed and as they happen to influence the result in opposite directions they do not appreciably affect the analytical results For urine work ordinary 90 per cent ethyl alcohol might, as a matter of fact, be substituted for the ether-alcohol mixture recommended above but, since we have satisfied ourselves that uric acid is rather more soluble in it than in the ether-alcohol mixture, we prefer the latter

To the washed residue in the bottom of the beaker is next added water (5–10 cc) and a drop of saturated sodium carbonate solution, and the mixture is shaken or stirred so as to secure complete

solution of the uric acid To the solution is finally added, first, 2 cc of the uric acid reagent and then 20 cc of saturated sodium carbonate solution The resulting blue solution is transferred to a 100 cc measuring flask, diluted with water up to the mark and the intensity of the color is determined by means of a Duboscq colorimeter The standard solution for comparison is obtained by treating 1 mgm of uric acid in lithium carbonate solution with 5–10 cc of water, 2 cc of the uric acid reagent and 20 cc of sodium carbonate solution, and the whole made up to 100 cc in a volumetric flask

The uric acid reagent and carbonate solution should be added as nearly simultaneously as practicable to both the unknown and the standard because the solutions obtained deteriorate rather rapidly (sufficiently rapidly for example to produce an unmistakable difference in color in fifteen minutes) This deterioration is of no consequence to the result if the solutions are made at about the same time, for the known and the unknown will deteriorate at practically the same rate It is therefore perfectly feasible to make several uric acid determinations by the help of the same standard solutions provided the uric acid reagent has been added to all of them at about the same time (*i e*, within three to five minutes).

As stated above, the color obtained from 1 mgm of uric acid is used as a standard Unfortunately uric acid solutions do not keep very well though when properly made they last for about a week, certainly for several days To obtain such stable uric acid solutions it is necessary to add only just enough lithium carbonate to dissolve the uric acid in the course of about an hour A 0 4 per cent lithium carbonate solution is very convenient for the purpose Each cubic centimeter of such a solution dissolves 10 mgm of uric acid For a standard solution we therefore weigh out 250 mgm of Kahlbaum's uric acid, transfer it to a 250 cc volumetric flask by means of 25–50 cc of water, then add 25 cc of the lithium carbonate solution and shake at intervals for an hour before diluting with water

The preparation of such a standard uric acid solution once a week is not much of a task if in the course of that week a number of uric acid determinations are to be made A standard solution which remains reliable only for a week is however far from an ideal standard

We have devoted a great deal of time trying to find a more permanent standard, for it would manifestly be a great advantage to have it always on hand for occasional uric acid determinations

Riegler adopted colored glasses for the purpose, but so far we have been unable to find any which are serviceable, because they transmit too much or too little light We have also investigated a large number of substances which give blue solutions, including the aniline dyes, but here again they are entirely too bright to be satisfactory with the ordinary Duboscq colorimeter

We have also experimented with a large number of substances (polyphenol derivatives) which give the blue color with the uric acid reagent but curiously enough nearly all give blue solutions which are less bright than the blue given by uric acid, besides having a more greenish shade

It therefore seems almost hopeless to get any standard solution of keeping quality capable of giving exactly the same color as that obtained with uric acid A good, though by no means ideal standard solution, was finally found in the uric acid reagent itself It was found that an excess of the reagent used with a minute quantity of uric acid gives the same color as is obtained when an excess of uric acid is added to a minute quantity of the uric acid reagent, and as the latter seems to keep indefinitely, a part of it can be set aside and standardized against a known uric acid solution and will then be found to be very serviceable for occasional uric acid determinations

When employed as a standard the uric acid reagent is used as follows A small pinch (a few milligrams) of uric acid is thrown into a 100 cc measuring flask and is dissolved by the addition of 20 cc of saturated sodium carbonate solution (No attention need be paid to the rapidity or completeness with which the uric acid dissolves) To this solution is then added 1 cc of the uric acid reagent or a corresponding quantity of a uric acid reagent previously diluted with water The blue solution is diluted to 100 cc and its color value is determined by comparison with the color given by 1 mgm of uric acid when treated in the usual manner with an excess (2 cc) of the uric acid reagent

Setting the prism of the known uric acid solution at 2— mm , it will be found that 1 cc of the reagent treated with an excess of uric acid as described will read somewhere between 9 and 12 mm Suppose it reads at the latter figure, then setting it at 12 when

using it as a standard for the determination of uric acid in an unknown solution or in urine is the same as using 1 mgm. of uric acid and setting the standard at 20. To illustrate: If an unknown urine against this new standard gave a color of the same intensity as the standard named, it would contain $\frac{20}{12}$ or 1.666+ mgm. of uric acid in the volume of urine taken for analysis; if the unknown read 11 mm. with the standard at 12, it would contain $\frac{20}{11}$ or 1.82 mgm. of uric acid.[5] The chief drawback encountered in using the uric acid reagent as a standard is that the color produced fades more rapidly than the color produced when the reaction is made in the usual way with an excess of the reagent. It is therefore necessary to make the reaction as nearly simultaneously as possible in the unknown and in the standard and then to read the color promptly, *i. e.*, within five minutes. For each unknown to be determined a fresh reaction must be made with the standard. When a series of uric acid determinations are to be made it is *therefore in most cases best to use uric acid rather than the uric* acid reagent as a standard.

The uric acid values recorded below for twenty normal urines show that the new colorimetric method gives substantially the same figures as are obtained by the Folin-Shaffer modification of Hopkin's method. The figures given in the fourth column represent the uric acid plus all other substances in urine which give a blue color with our uric acid reagent. As indicated in the table, we regard these as being chiefly polyphenols.

Since the reaction for uric acid which is here used as a basis for its determination is more delicate than any other known reaction and is perfectly adaptable for quantitative work, it is clear that it ought to prove eminently suitable for the determination of such small quantities of uric acid as may exist in blood. A new method for the determination of uric acid in blood, based on the principles outlined in this paper, will be described in the next number of this *Journal*.

[5] It doubtless will occur to some that it would be more convenient to dilute the uric acid reagent so that a suitable volume should have the same chromophoric value as the solution obtained from 1 mgm. of uric acid. There is however nothing to be gained by such a procedure. It is just as easy to set the standard somewhere between 9 and 12 as at 20. Moreover, the more concentrated color corresponding to 1 cc. of the uric acid reagent fades less rapidly than do weaker solutions.

Uric acid in urine (grams per liter)

NO	FOLIN-SHAFFER METHOD	COLORIMETRIC METHOD	URIC ACID AND POLYPHENOLS	SPECIFIC GRAVITY OF URINE
1	0 16	0 19	0 26	1 012
2	0 49	0 50	0 59	1 027
3	0 50	0 49	0 67	1 027
4	0 41	0 41	0 60	1 017
5	0 49	0 49	0 60	1 027
6	0 59	0 58	0 81	1 027
7	0 57	0 56	0 72	1 030
8	0 41	0 40	0 54	1 027
9	0 42	0 41	0 51	1 027
10	0 41	0 43	0 54	1 022
11	0 13	0 16	0 40	1 012
12	0 36	0 36	0 52	1 022
13	0 67	0 67	0 75	1 027
14	0 28	0 28	0 45	1 019
15	0 23	0 23	0 41	1 018
16	0 31	0 32	0 39	1 021
17	0 78	0 74	0 87	1 030
18	0 60	0 62	0 75	1 029
19	0 74	0 74	1 01	1 028
20	0 51	0 50	0 63	1 025

THE METABOLISM OF ENDOGENOUS AND EXOGENOUS PURINES IN THE MONKEY[1]

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(Received for publication, November 1, 1912)

The introduction by Wiechowski[2] of an accurate method for the determination of allantoin led very quickly to the recognition of that substance as a constant constituent of the urine of mammals, to be included with uric acid and the purine bases among the regular products of endogenous purine metabolism It appears practically certain that in no mammalian urine is any one of these three stages in the progressive catabolism of the purine nucleus entirely unrepresented As for the relative importance of each, it is found to vary with the species Some animals excrete uric acid in greater abundance than purine bases,[3] while for others the reverse is true In most cases the rôle of these two fractions combined is greatly subordinate to that of allantoin Thus in the dog[4] allantoin accounts for 93 to 97 per cent of the total purine nitrogen eliminated, and a ratio of the same order has been established for the cat,[5] the rabbit,[5] the pig[6] and the coyote [7] In another group of animals,

[1] The experimental matter contained in this paper was presented in part at the Joint Meeting of the American Society of Biological Chemists and the Biological Section of the American Chemical Society, Washington, December, 1911, and in part at the Eighth International Congress of Applied Chemistry, New York, September, 1912

[2] Wiechowski *Beitr z chem Physiol* , xi, p 109, 1908

[3] This is known to be true for the horse (Schittenhelm and Bendix *Zeitschr f physiol Chem* , xlviii, p 140, 1906) and the pig (Schittenhelm and Bendix *loc cit* , Schittenhelm *Zeitschr f physiol Chem* , lxvi, p 53, 1910, Mendel and Lyman this *Journal*, viii, p 115, 1910)

[4] Wiechowski *loc cit* , Schittenhelm *Zeitschr f physiol Chem* lxii, p 80, 1909, Hirokawa *Biochem Zeitschr* , xxvi, p 441, 1910

[5] Wiechowski *loc cit*

[6] Schittenhelm *loc cit* 1910

[7] Hunter and Givens this *Journal*, viii, p 449, 1910

exemplified by the horse[8] (and as will appear, the monkey), the predominance of allantoin is less pronounced One species, namely man, occupies in this respect an altogether exceptional position Human urine is characterized by its high content of uric acid, bases are present in relatively small amounts, while allantoin has been detected in the merest traces [9]

It is natural to inquire whether the specific peculiarities thus manifested permit of being systematized Particularly is one tempted to ask whether in this province similarities of physiological reaction correspond with genetic affinities and whether among the variations encountered it is possible to trace any evolutionary sequence With the purpose of collecting data which may ultimately afford an answer to such questions we have undertaken the systematic examination of a number of mammalian urines not hitherto studied from this point of view

The present paper deals with one species only, but it is representative of a group to which a special interest attaches, and it has been made the subject of rather more extended observation and experiment than lay in our original plan The apparently unique behavior of the human organism suggested an early attempt to ascertain the character of the purine-allantoin excretion in man's nearest relatives Material from the higher apes we have not been fortunate enough to have at our disposal but about two years ago we were able to take up the problem in regard to the monkey It is true that even at that time data on the subject were not altogether lacking Wiechowski[10] had examined one sample of monkey urine (species not stated), and had found it to contain as much as 0 1 gram of allantoin in 100 cc , uric acid was absent, purine bases were apparently not determined To the general question involved an important contribution had also been made by Wells [11] In a study devoted particularly to the purine enzymes of the tissues of *Macacus rhesus* he demonstrated that the liver of this

[8] Wiechowski *Biochem Zeitschr* , xix, p 368, 1909

[9] Wiechowski *loc cit* , 1909 and *Biochem Zeitschr* , xxv, p 431, 1910, Schittenhelm and Wiener *Zeitschr f physiol Chem* , lxiii, p 283, 1909, Ascher *Biochem Zeitschr* , xxvi, p 370, 1910, Fairhall and Hawk *Journ Amer Chem Soc* , xxxiv, p 546, 1912

[10] Wiechowski *loc cit* , 1908

[11] Wells this *Journal*, vii, p 171, 1910

monkey e\hıbıts *ın vıtro* a hıgh degree of urıcolytıc power In spıte of thıs he was unable, eıther by the Wıechowskı method or by the older one of Poduschka,[12] to ısolate allantoın from the urıne of the anımals employed At the most ıts presence was rendered probable Urıc acıd was encountered on one occasıon only, and was then ascrıbed to the effects of tuberculous dısease The total purıne e\cretıon was exceedıngly small

Publıshed observatıons on the poınt specıally ınterestıng us were, ıt ıs seen, not only few ın number but rather ımperfectly concordant, and we felt justıfied ın pursuıng the questıon ındependently In a first communıcatıon[13] we confirmed Wıechowskı as to the presence of allantoın ın monkey's urıne, Wells as to the smallness of the total purıne output and both as to the normal absence of urıc acıd But the few analyses reported were made on mıxed samples obtaıned at ırregular ıntervals from two monkeys of dıfferent specıes They possessed therefore lıttle more than a qualıtatıve value We have sınce subjected one of the anımals to a serıes of quantıtatıve studıes An abstract of our earlıer results had already been publıshed,[14] when a fresh contrıbutıon to the subject appeared from the pen of Wıechowskı[15] In thıs he states the conclusıon, fully ın accord wıth our own observatıons, that ın monkeys the prıncıpal end product of purıne metabolısm ıs allantoın, whıle he reports ın addıtıon the extremely ınterestıng dıscovery, that thıs substance ıs absent from the urıne of the chımpanzee Although our program, as orıgınally planned, ıs not yet completed, the appearance of Wıechowskı's paper leads us to render wıthout further delay a detaıled account of some of our experıments

[12] Poduschka *Arch f exp Path*, xlıv, p 59, 1900

[13] Hunter and Gıvens Proc Amer Soc Bıol Chem, December, 1910, thıs *Journal*, ıx, p xvı, 1911

[14] Hunter and Gıvens Proc Amer Soc Bıol Chem, December, 1911, thıs *Journal*, xı, p xxxıx, 1912 Further data, some of whıch also are ıncorporated ın the present paper, formed the subject of another brıef communıcatıon see *Orıg Comm Eıghth Intern Congr of Applıed Chem*, xıx, p 149, September, 1912 Thıs was already ın prınt before we became aware of the exıstence of Wıechowskı's recent artıcle

[15] Wıechowskı *Prager med Wochenschr*, 1912, p 275 The orıgınal paper ıs not wıthın our reach, we quote from an abstract ın *Zentralbl f Bıochem u Bıophys*, xııı, p 661, September, 1912

SUBJECT AND METHODS

The subject was an adult female "guenon" monkey (*Cercopithecus callitrichus*),[16] weighing 4 7 to 4 8 kgm , of very active habit and lively though uncertain temper When under observation it was confined in a metabolism cage of the ordinary type and was maintained upon a diet limited to milk, peanuts and bananas, with the occasional addition of some common salt Water, as a separate item, was neither given nor apparently desired

We hoped at first to be able to maintain the animal in uninterrupted nitrogenous equilibrium, but the rather capricious nature of its appetite forced us to abandon the attempt The diet was at least adequate, the body weight increased rather than diminished during the experiments and except possibly towards the end of the second period of confinement the monkey was in perfect condition

The oral administration of sodium nucleate and of allantoin was effected by dissolving them in the minimum amount of water and adding the solution to the morning draught of milk During the day successive portions of the milk ration were offered in the same polished nickel dish, quantitative ingestion of the dose was thus in every case assured[17] The subcutaneous injections were made from an elevated burette into the loose tissue round the loins They produced no perceptible local or general effects

The urine was collected in forty-eight-hour periods The temper of the animal made the use of the catheter out of the question Fortunately she could be induced to micturate at any time by transferring her from one cage to another, and by taking advantage of this it was possible to delimit each period's excretion with an error of less than twenty minutes As the urine was frequently alkaline when passed, it was received in a flask containing acetic acid Putrefaction was prevented by the liberal use of thymol Each sample, as collected, was made up along with the cage washings

[16] The species was identified for us by Dr H D Reed of the Department of Vertebrate Zoology in this University To Dr Reed we desire to take this opportunity of expressing our indebtedness for the service

[17] On a few occasions after the administration of sodium nucleate a part of the last portion of milk for the day was refused This was hydrolyzed with 5 per cent sulphuric acid and tested for purines by the method of Krüger and Schmid No purines were ever detected

to a round volume of 1000 cc All the analyses were commenced immediately

Uric acid and *purine bases* were determined in a 400 cc portion The method employed was that of Kruger and Schmid[18] with these variations, that the use of MnO_2 was omitted, that the filtrate and washings from uric acid (when present) were not allowed to exceed 25 cc and that the correction for solubility of uric acid was not applied It follows that the figures under "purine bases" may, in the reported absence of uric acid, and do, in its presence, include a trace of the latter The error involved is not greater than 1 mgm for each two-day period

Allantoin was estimated by the method of Wiechowski Its application to the urine of our monkey proved to be not altogether a simple matter In fact in the first form described by its author it yielded us no allantoin whatever The difficulty lay, as afterwards appeared, partly in the small amount of allantoin actually excreted—less than 0 05 gram daily—and partly in the presence of substances which to all the precipitants employed reacted exactly like allantoin itself These obstacles were finally overcome by a procedure essentially the same as that employed by Wiechowski with human urine[19]

Two-fifths (400 cc) of each urine sample, acidified with sulphuric acid, was treated with the requisite amount (about 15 cc) of 50 per cent phosphotungstic acid The phosphotungstates were separated on a kieselguhr filter[20] The filtrate was neutralized with sodium hydroxide and treated, after the usual routine, first with 20 per cent basic lead acetate and then with a saturated solution of acetate of silver Every precipitate, especially the very heavy lead one, was thoroughly washed with cold water and the washings were added to the filtrate The final bulk of the liquid was consequently very considerable (about 1200 cc) It was carefully neutralized with sodium hydroxide and subjected to a partial precipitation with 20 per cent mercuric nitrate 8 cc of this reagent were all that were required to completely precipitate the allantoin, 5 cc of the filtrate invariably gave an immediate reaction with one drop of a 0 1 per cent solution of allantoin

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[18] Kruger and Schmid *Zeitschr f physiol Chem* , xlv, p 1, 1905

[19] Wiechowski *loc cit* , 1909

[20] The kieselguhr filter recommended by Wiechowski was employed not only here, but also in filtering the very fine silver precipitate of a later stage of the process It proved to be in these and many other situations exceedingly efficient for the retention of fine suspensions, but we are not without suspicion that its use was at least partly responsible for the ash content of our allantoin (see later)

In two of these (numbers 38 and 41) the effect of feeding sodium nucleate during the preceding forty-eight hours may still be traced For five others the record is imperfect There remain twenty-three "normal" periods, which, since the regular diet could have contained no appreciable quantity either of purines or of allantoin, may be taken to represent the endogenous excretion of the substances we are considering

TABLE I

PERIOD	GRAMS NITROGEN IN 48 HOURS					ALLANTOIN N IN PER CENT OF TOTAL PURINE-ALLANTOIN N	REMARKS
	Total	Allantoin	Uric acid	Purine bases	Total purine-allantoin N		
1	3 59		none	0 009			
2	3 81	0 031	none	0 009	0 040	78	
3	3 64	0 031	none				
4	*3 09	0 045	none	0 009	0 054	83	0 5 gram sodium nucleate *per os*
5	3 18	0 027	none	0 008	0 035	77	
6	3 59	0 047	trace	0 007	0 054	87	0 5 gram sodium nucleate *per os*
7	*3 41	0 029	none	0 010	0 039	74	
8	*3 28	0 056	none	0 010	0 066	85	1 0 gram sodium nucleate *per os*
9	3 28	0 029	none	0 009	0 038	76	
10	3 56	0 043	none	0 013	0 056	77	1 0 gram sodium nucleate *per os*
11	3 44	0 032	none	0 010	0 042	76	
12	3 78	0 065	0 004	0 011	0 080	81	2 0 gram sodium nucleate *per os*
13	3 43	0 029	none	0 012	0 041	71	
14	3 52	0 029	none	0 010	0 039	74	

* Rations not entirely consumed

The average excretion of purine nitrogen by the feces was for periods 1–3, 0 029, 4–6, 0 028, 7–10, 0 022, and 11–14, 0 025 gram

The *total purine-allantoin* output for these periods is a nearly constant quantity Expressed in terms of nitrogen, it lies on all but five occasions between the narrow limits of 38 and 42 mgm and it has an average value over all of 39, or, in twenty-four hours, 19 5 mgm This is a little more than 1 per cent of the total nitrogen excretion The endogenous purine metabolism of the monkey

TABLE II

PERIOD	GRAMS NITROGEN IN 48 HOURS					ALLANTOIN N IN PER CENT OF TOTAL PURINE-ALLANTOIN N	REMARKS
	Total	Allantoin	Uric acid	Purine bases	Total purine-allantoin N		
15	3 39		none	0 010			
16	3 50	0 030	none	0 010	0 040	75	
17	3 60	0 027	none	0 011	0 038	71	
18	3 47	0 030	none	0 011	0 041	73	
19	3 64	0 030	none	0 011	0 041	73	
20	3 74	0 031	none	0 011	0 042	74	
21	*3 29	0 030	none	0 010	0 040	75	
22	*3 30	0 028	none	0 011	0 039	72	0 1295 gram allantoin *per os*
23	3 44	0 029	none	0 011	0 040	73	
24	3 52	0 042	none	0 010	0 052	81	0 2765 gram allantoin *per os*
25	3 51	0 028	none	0 012	0 040	70	
26	3 71	0 050	none	0 012	0 062	81	0 2718 gram allantoin *per os*
27	*3 45	0 027	none	0 010	0 037	73	
28	*3 37	0 089	none	0 010	0 099	90	0 1988 gram allantoin subcutaneously
29	*3 15	0 024	none	0 009	0 033	73	
30	3 41	0 029	none	0 007	0 036	81	
31	*3 28	0 054	none	0 011	0 065	83	0 0946 gram allantoin subcutaneously
32	*3 15	0 029	none	0 012	0 041	71	
33	*3 50	0 025	none	0 014	0 039	64	
34	*3 42	0 054	none	0 013	0 067	81	0 0963 gram allantoin subcutaneously
35	*2 89	0 027	none	0 012	0 039	69	
36	*2 69	0 026	none	0 012	0 038	68	Diarrhoea
37	*3 53	0 035	0 004	0 011	0 050	70	2 0 grams sodium nucleate *per os*
38	*3 42	0 024	trace	0 012	0 036	67	
39	3 60	0 020	none	0 010	0 030	67	
40	3 75	0 045	0 004	0 013	0 062	73	2 0 grams sodium nucleate *per os*
41	*3 40	0 025	0 001	0 012	0 038	66	
42	*3 20	†(0 019)	none	0 014			

* Rations not entirely consumed
† The allantoin determination of this period was lost. The figure given was obtained on period 43

Average excretion of purine nitrogen by the feces for periods 15–21, 22–36, 0 011, 37–42, 0 011 gram

is evidently pitched upon a comparatively low level It happens that we can compare our subject in this respect with a dog of identical weight (4 7 kgm), this animal, as reported by Hirokawa,[23] on a diet all but free from purines and excreting 1 64 grams of total urinary nitrogen, eliminated daily 0 159 gram of purine + allantoin nitrogen This is eight times as much as the monkey

Uric acid could be detected in none of the "normal" urines, if this does not positively prove its absence, it shows at least that any quantity actually eliminated must have been excessively minute

Purine bases on the other hand are excreted regularly in relatively considerable amount The nitrogen present in this form varies between 7 and 14 mgm Each extreme is an exceptional figure, on the whole the purine base excretion exhibits great regularity, seventeen of our twenty-three normal values lying between 10 and 12 mgm The average of all is 10 5 (daily, 5 25) mgm of nitrogen The monkey, it appears, is one of the few animals known to excrete purine bases in greater quantity than uric acid

For endogenous *allantoin* the lowest nitrogen value recorded is 20 mgm , the highest, 32 Here again a much greater approach to constancy is made than these isolated figures would indicate There is reason to believe that the minimum (period 39) was reached as an indirect result of previous feeding with sodium nucleate, the two nearest to it are 24 and 25 (periods 29 and 33), and even these are in all probability similarly related to the allantoin injections[24] Be this as it may, there are eighteen periods for which the allantoin nitrogen falls between 27 and 31 mgm The average of the whole twenty-three is 28 If for the reasons stated we neglect the three lowest, the average is 29 This means a daily excretion of 14 5, the equivalent of 0 041 gram of allantoin

These perfectly consistent results confirm and are confirmed by the reports of Wiechowski upon what were doubtless other species of monkey They are entirely in line with the demonstration by Wells of the uricolytic capacity of the *Macacus* liver They afford no evidence whatever of an approach to the human type. From the point of view of endogenous purine metabolism, the monkey decidedly ranks with the lower mammals Yet the urin-

[23] Hirokawa *loc cit*

[24] See the second last paragraph of this paper

ary picture presents peculiarities A reference to the seventh column of Tables I and II shows that the share taken by allantoin in the sum of purine derivatives is not precisely that which is typical of the majority of animals The figure expressing it—what we may call the "allantoin ratio"—may in "normal" periods be as low as 64 or as high as 81 per cent but it generally remains somewhere between 70 and 78 It never attains the high values recorded for species so divergent as the dog and the pig On the average only 73 per cent of the total nitrogen of endogenous purine origin takes the form of allantoin, as much as 27 per cent is left for the purine fraction This state of affairs resembles the condition found by Wiechowski to be characteristic for the horse, here the "allantoin ratio" was in one case 50, in another 79 [25] Curiously enough the urine of this animal presents another similarity to the monkey's in the great preponderance of purine bases over uric acid According to a few analyses of sheep's urine made in this laboratory—upon which, however, we do not at present place too much reliance[26]—the "allantoin ratio" of this animal also is rather low while its purine fraction is about equally divided between the bases and uric acid A full explanation of these interesting relations is not yet possible

The fate of sodium nucleate and allantoin in the organism of the monkey

We have thus far taken for granted that in the purine metabolism of our monkey no other products actually arise than those considered It seemed to us desirable to test the validity of this assumption The widely held conviction that man, in regard to this very point, offers a notable contrast to the lower mammals, would add a special interest to the outcome of such a test In pursuance of this idea we attempted, in the first place, to ascertain the fate of sodium nucleate administered by the mouth

[25] Wiechowski *loc cit*, 1909 Wiechowski did not determine the purine base content of his horse urines If this were as high as Schittenhelm and Bendix (*loc cit*) found it in other samples, the allantoin ratio in our sense would be lower still

[26] They were made upon the urine of thyroidectomized sheep, and may therefore represent ratios that are pathological They will be published later in another connection

The sodium nucleate used was obtained from Merck It contained in the air-dried state 13 99 per cent of nitrogen Its purine content was determined by twice repeated precipitation with copper sulphate and sodium bisulphite after hydrolysis with 3 per cent sulphuric acid, duplicate analyses gave 8 67 and 8 69, average, 8 68 per cent of purine nitrogen

The quantity administered was so chosen as to be not altogether out of proportion with the extent of the endogenous metabolism The smallest dose contained rather less purine-ring nitrogen than the combined allantoin and purines of the normal urine and the largest not four times this amount Bigger ones, it was feared, might by bringing about of themselves an abnormal condition defeat the very object of their administration Certain experiments of Hirokawa, in which the natural relation between uric acid and allantoin was altered by repeated administration of sodium nucleate,[27] afford evidence that this is a consideration to be borne in mind Indeed our own maximal dose, small as it was in comparison, was not always without perceptible after-effect upon the regular endogenous metabolism [28]

In Tables I and II there are recorded seven experiments with sodium nucleate Inspection of the data for fecal purines makes it almost certain that absorption was in every case complete An increase of urinary allantoin follows every dose After the larger ones uric acid also is excreted in measurable amounts An effect upon the purine bases might, to judge from their share in the endogenous excretion, have reasonably been expected but none is with certainty discernible, a slight rise in this fraction does sometimes (periods 10 and 40) take place but at other times there is a fall and the rise is paralleled on other occasions (for example 33 and 42) quite unconnected with nucleate feeding The net result upon the "allantoin ratio" is to raise it somewhat above its earlier level Forty-eight hours generally suffices for the complete elimination of the catabolic products But on two occasions (38 and 41) the excretion of uric acid undoubtedly stretched into the succeeding period, and one may with probability assume that a portion of the allantoin accompanying it was likewise derived from the exogenous source This indicates for the monkey, as compared with the dog, a rather slow rate either of absorption or of elimination

The experiments, as was to be expected, reveal allantoin as an important product of the exogenous as well as the endogenous metabolism of purines They yield in addition positive evidence of the appearance of uric acid as its precursor In their quantita-

[27] Hirokawa *loc cit*

[28] This is discussed in the last paragraph but one of the paper

tive aspects they are not so satisfactory In Table III the results are so arranged as to show what fraction of the purine nitrogen fed was actually accounted for

As the norm from which to estimate the actual increase of allantoin in any given experiment we have taken the mean between the output of the period immediately preceding and that of the earliest subsequent period in which no exogenous excretion is detectable (The latter, as we have noted, is generally the period immediately after the experimental one but sometimes the next again) The intake is measured as four-fifths of the total purine nitrogen administered, that fraction only being contained in the purine ring and available for transformation into uric acid or allantoin

We recover, in ascertained combinations, with small doses about half,[29] sometimes as little as an eighth with larger ones, and as the

TABLE III

PERIOD	PURINE-RING N FED	MGM OF NITROGEN RECOVERED			PERCENTAGE RECOVERY
		As uric acid	As allantoin	Total	
4	35	0	16	16	46
6	35	trace	19	19	54
8	70	0	27	27	39
10	70	0	12	12	17
12	140	4	35	39	28
37	140	4	13	17	12
40	140	5	30	35	25

average of all only 32 per cent In face of the almost certainly complete absorption of the nucleate, how is the deficit to be explained? Perhaps the first possibility that suggests itself is that allantoin may be no end product, but liable itself to catabolic changes It is true that metabolic processes do not often exhibit the irregularity that would then seem to characterize the destruction of allantoin Nevertheless we felt bound to submit the possibility to the test of experiment On three occasions allantoin was administered by mouth, on three others, by subcutaneous injection The same principles as before were applied to the selection of doses

[29] In an earlier report already referred to (Hunter and Givens *loc cit*, 1912) we alleged a recovery, after the first dose, of 90 per cent, this statement involved, as we have since discovered, a gross error in calculation

and to the interpretation of the data obtained The results are brought together in Table IV

Between the two sets of allantoin experiments with their almost directly contradictory results we have no hesitation in selecting the injections as the decisive ones In these alone are we absolutely certain that the substance entered unaltered into the organism, and it is the fate of allantoin within the organism, and that only, that concerns us It has been objected to the parenteral modes of administration that they may induce disturbances of general metabolism so profound as to obscure the true interpretation of results [30] The objection is doubtless in many cases sufficiently well grounded, but in the present one no greater disturbance was noticed than might have been produced by the injection of so

TABLE IV

PERIOD	METHOD	MILLIGRAMS ALLANTOIN N		PERCENTAGE RECOVERY
		Administered	Recovered	
22	oral	45 8	0	0
24	oral	98 0	13	13
26	oral	96 3	22	22
28	subcutaneous	70 4	63	90
31	subcutaneous	33 5	25	75
34	subcutaneous	34 1	28	82

much water The feeding experiments on the other hand are attended by numerous uncertainties The warmth and alkaline reaction of the intestinal contents are of themselves likely, according to Wiechowski,[31] to bring about the rapid destruction of allantoin Add to this the slight solubility of the substance and the possible action of bacteria, and it would not be at all surprising if none of the first small dose we gave had ever reached the blood stream It is to such factors that we incline at present to attribute the poor recovery of ingested allantoin

However this may be, the results of the injection experiments are perfectly definite Small doses of allantoin, subcutaneously introduced, are recoverable from the urine of the next forty-eight

[30] Cf Schittenhelm *loc cit*, 1909
[31] Wiechowski *loc cit*, 1910

hours almost as completely as if they had been directly dissolved therein It seems impossible to escape from the conclusion that, if the monkey tissues possess any capacity at all for the destruction of allantoin, it is so poorly developed as to have but little practical significance

The results of our allantoin experiments with the monkey are paralleled by those reported upon other species After ingestion there have been recovered widely varying, often very low, proportions in the dog, 70 (Minkowski[3]), 90 (Poduschka[33]) and 31 per cent (Levene and Medigreceanu[34]), in the pig, nearly 100 (Schittenhelm[35]), in man, 17 (Minkowski[36]), 30–50 (Poduschka[37]), 34 (Wiechowski[38]) and 30–38 per cent (Schittenhelm and Wiener[39]) Subcutaneous administration has been seldom employed once by Schittenhelm[40] on the pig with quantitative recovery and thrice by Wiechowski[41] on man with yields up to 88 per cent [4]

Most writers are in fact at one in regarding allantoin as a terminal and not merely an intermediary product of metabolism Our contribution to the question serves but to emphasize the correctness of this view To explain the deficit of our sodium nucleate experiments we must look then to other factors than allantoin destruction What these actually are, it is as yet impossible to say But there are certain considerations, which appear to us to suggest the direction in which they are to be sought

There is among recent investigators a pretty general agreement that, when nucleic acid is fed to an animal like the dog, its constituent purines can be quantitatively accounted for (or nearly so) by the excess of allantoin, uric acid and bases eliminated during the

[3] Minkowski *Arch f exp Path*, xli, p 375, 1898
[33] Poduschka *loc cit*
[34] Levene and Medigreceanu *loc cit*
[35] Schittenhelm *loc cit*, 1910
[36] Minkowski *loc cit*
[37] Poduschka *loc cit*
[38] Wiechowski *Arch f exp Path*, lx, p 185, 1909
[39] Schittenhelm and Wiener *loc cit*
[40] Schittenhelm *loc cit*, 1910
[41] Wiechowski *Arch f exp Path*, lx, p 185, 1909
[42] Schittenhelm and Seisser (*Zeitschr f exp Path*, vii, p 116, 1909) injected allantoin intravenously into the rabbit Only an abstract of their paper is available (*Biochem Zentralbl* ix, p 746) and the quantitative details are not there given The above list includes, we believe, all other experiments in which the recovery was quantitatively determined

next twenty-four hours Schittenhelm,[43] for example, reports recoveries of 88–102, Hirokawa,[44] of 72–88 per cent We may therefore take it as demonstrated that in the dog no other products of purine catabolism need be looked for It appears at least equally well established that man, after the ingestion and undoubted absorption of nucleate of sodium, excretes as uric acid only a fraction (according to the particularly careful experiments of Frank and Schittenhelm[45] from 5 to 41 per cent) of the purine intake, while no perceptible increase of the insignificant allantoin output assists in making good the deficit [46]

A vehement controversy centers round the explanation of this difference in behavior According to what is probably at present the prevailing view,[47] uric acid is in man, as much as in the lower animals, an intermediary product only, and its destruction is accomplished in his case by a peculiar type of uricolysis leading not to or even through allantoin, but, by steps not yet elucidated, to urea Another school of opinion denies to human tissues every kind of uricolytic power, regards uric acid therefore as a terminal product, and attributes the observed deficit after nucleate feeding to destruction of the purine ring before absorption [48]

We do not propose to enter here into the merits of this controversy What is of interest to us is that in response to the enteral administration of sodium nucleate the monkey reacted, not like the animal with which its high allantoin excretion would associate it, but like man It is hardly likely that we are dealing here with two unrelated processes leading to the same exceptional result It may be assumed with greater probability that that theory of the fate of ingested nucleic acid which is ultimately found to hold good for the human animal will equally fit the monkey If the experimental facts require us to postulate for the former a particular kind of uricolytic enzyme, that enzyme is probably active in the latter also We should be led, then, to the unexpected con

[43] Schittenhelm *loc cit*, 1909

[44] Hirokawa *loc cit*

[45] Frank and Schittenhelm *Zeitschr f physiol Chem*, lxiii, p 269, 1909

[46] Schittenhelm and Wiener *loc cit*

[47] Cf Brugsch and Schittenhelm *Der Nucleinstoffwechsel*, Jena, 1910, pp 41–45, where the literature of the controversy will be found

[48] See the papers of Wiechowski already cited, also Sivén *Arch f d ges Physiol*, cxlv, p 283, 1912

clusion that the monkey disposes of intermediary purines by two independent mechanisms the one peculiar to the lower mammals and leading to allantoin, the other ending in urea and encountered in no other species save man This, if true, would be a striking illustration of the monkey's philogenetic relations, but it hardly bears the stamp of probability It is easier to believe that the undeniable destruction of purine nuclei, which we observed, was really accomplished in the monkey's intestine before absorption had taken place If this happens in the monkey's intestine, it may also happen in man's, and while our experiments have no direct bearing on the problems of human metabolism, we think that they afford some indirect evidence against the assumption of an extensive uricolysis in the human organism

One curious circumstance deserves perhaps more particular notice than we have yet bestowed upon it If the data of Table I be reviewed, it will be observed that in no case there recorded does the administration of sodium nucleate produce any effect other than the immediate one of an increase in the total purine metabolism [49] There is after every dose a prompt return to the same "normal" level of allantoin excretion It is otherwise with the experiments of Table II Here may be noted from period 27 onwards an almost uninterrupted sinking of the "normal" figures for allantoin Each rise of the output, whether resulting from allantoin injection or from nucleate feeding, is followed, more or less immediately, by a fall below the previous standard Most striking are the drop from 27 to 24 mgm after the first allantoin injection, from 29 to 25 after the second and from 26 to 20 after the first dose of nucleate The summation of these effects brings the allantoin nitrogen finally down to two-thirds of its original value This phenomenon, in relation at least to nucleate feeding, has already been noticed by Schittenhelm in experiments upon the dog and the pig [50] Our observation, that it may follow simple allantoin injections, suggests that in both cases it is the temporary excess of cir-

[49] It may be worth recording that we determined regularly during this series the excretion of ammonia and creatinine and that these exhibited no single departure from a very narrow range of values, the ammonia nitrogen varied between 0 039 and 0 049 gram, creatinine nitrogen between 0 123 and 0 134, nucleate administration having no perceptible effect upon either

[50] Schittenhelm *loc cit*, 1909 and 1910

culating allantoin that in some way is responsible But the same remarkable after-effect has been seen to follow the administration of histidine hydrochloride, which has no relation at all to the production of allantoin[51] The exact significance of the lowered output remains obscure It was not accompanied in the monkey by any uniform variation of the purine base excretion

SUMMARY

In the urine of the guenon monkey (*Cercopithecus*) allantoin accounts for about 73 per cent of the nitrogen arising from the catabolism of endogenous purines The remainder appears principally as purine bases, uric acid being practically absent when the diet is purine-free The allantoin is a true end product When purines are ingested in the form of sodium nucleate, the allantoin output is increased and uric acid may appear as an intermediate product Only 12 to 54 per cent of the total purine intake is thus accounted for The deficit is probably the result of decomposition prior to absorption There is no evidence in this monkey of an approach to the human type of nuclein metabolism

(Experiments upon the fate of individual purines in the monkey organism are now in progress, and will be reported upon in a later communication)

[51] See Abderhalden and Einbeck *Zeitschr f physiol Chem* , lxii, p 322, 1909, and Abderhalden, Einbeck and Schmid *ibid* , lxviii, p 395, 1910

ABSORPTION FROM THE STOMACH—A REPLY TO LONDON

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(Received for publication, November 12, 1912)

In our communication on Absorption from the Stomach[1] we gave figures which in our judgment leave no room for doubt concerning the absorption of protein digestion products from the stomach To E S London our results are evidently not at all convincing and in a recent number of the *Zeitschrift für physiologische Chemie*[2] he has endeavored to make clear several reasons why our results are inconclusive In view of the great amount of interesting experimental work done by London and his associates on the subject of absorption (by methods entirely different from ours) it is not at all strange that he should be unable to accept our conclusion, diametrically opposite as it is to the conclusion reached by himself

The three main points raised by London against our work are briefly discussed below

1 The increase in the non-protein nitrogen of the blood during our absorption experiments might be due to absorption from the intestinal tract of our fasting animals

This is to us an interesting argument in view of the fact that it heretofore has been practically impossible to demonstrate any increase in the non-protein nitrogen of the blood as a result even of carefully planned absorption experiments with the intestine As a matter of fact the absorption which we obtained from the stomach is more rapid than any absorption from the small intestine which we have observed as a result of ordinary feeding

[1] This *Journal*, xii, p 259

[2] *Zeitschr f physiol Chem*, lxxxi, p 283, 1912

Numerous experiments made in this laboratory have moreover shown that unless there is in the intestine a fair amount of material suitable for absorption, the non-protein nitrogen of the blood remains just about stationary during the short periods covered by our experiments Some such experiments made for other purposes have already been recorded See, for example, the two creatinine experiments[3] and the experiment on the absorption of tyrosine,[4] *in all of which virtually no increase in the non-protein nitrogen* of the blood was obtained

We would further call attention to the fact that urea is particularly rapidly absorbed from the stomach [5] London will have to concede that the accumulation of urea in the blood accompanying the disappearance of urea from the stomach can be due only to the absorption of urea from the stomach And having conceded this, there is, so far as we can see, no reason why the absorption of glycocoll, etc , should be less readily conceded

2 To London it seems strange that we should affirm that something was absorbed from Witte's peptone in the stomach, when we obtained only 40 mgm of non-protein nitrogen per 100 cc of blood, yet find no absorption of creatinine, although in one creatinine experiment the corresponding figure for the non-protein nitrogen of the blood was 53 mgm

The amount of the initial non-protein nitrogen in the blood is determined by the previous condition of the animal It is only the increase during the absorption experiment which proves anything In the creatinine experiment referred to[6] the non-protein nitrogen rose from 52 to 52 mgm , i e , nothing in ninety minutes *In the peptone experiment on the other hand the corresponding* figure rose from 34 to 40 during the same length of time

3 London finally falls back on the vague and all too common argument that the facts observed have anyhow no bearing on what happens normally Because dogs die in the course of a few days when the pylorus is closed by means of a ligature, London would insist that the absorption from the stomach of cats obtained in the course of a few minutes after the application of such a ligature

[3] This *Journal,* xii, p 262–3
[4] *Ibid* , xii, p 147–8
[5] *Ibid* , xii, p 263
[6] *Ibid* , xii, p 263

is to be explained as a pathological rather than as a physiological phenomenon We are disposed not to argue over this point but would only remark that a similar view can then be advanced against almost every experiment with animals, to say nothing of experiments of every description with isolated organs

It should be noted that London's results stand in a measure alone Tabler,[7] Salaskin,[8] Cohnheim,[9] Schewnert,[10] and Lang[11] all have reached the conclusion that nitrogenous products disappear from the stomach through direct absorption Taken in conjunction with the results of all these investigators ours might be regarded as only a verification (by a more direct method) which at the same time proves that the nitrogenous products absorbed from the stomach reach the circulation in non-protein form

Finally we wish to thank London for his courtesy in sending us an advance copy of his note on our work and to express our regret that he just now (as he has privately informed us) will be unable to publish further experimental work on the problem As we indicated in our original paper[12] London's experimental results are not necessarily inconsistent with ours His absorptions may have been hidden by excessive secretions into the stomach of various kinds of nitrogenous materials, while in our experiments (which anyhow were of relatively short duration) such secretion could not hide the accumulations in the blood

[7] *Zeitschr f physiol Chem*, xlv, p 185, 1905
[8] *Ibid*, lii, p 167, 1907
[9] *Ibid*, lviii, p 64, 1908
[10] *Ibid*, li, p 535, 1907
[11] *Biochem Zeitschr*, ii, p 225, 1906
[12] This *Journal*, xii, p 264

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A N Richards,
Secretary

ON THE INTENSITY OF URINARY ACIDITY IN NORMAL AND PATHOLOGICAL CONDITIONS

By LAWRENCE J HENDERSON AND WALTER W PALMER[1]

(*From the Chemical Laboratory, Massachusetts General Hospital*)

(Received for publication, November 2, 1912)

The researches of v Rohrer, Hober and one of us[2] have shown that both normally and pathologically the true acidity (concentration of ionized hydrogen) in human urine is subject to considerable inconstancy On the whole the variation is such as is *characteristic of a solution containing phosphoric acid and base* in which the latter ranges from that amount required to form monosodium phosphate to nearly that amount required to form disodium phosphate, and such fluctuation in the urinary phosphates is in fact the principal cause of the varying acidity, both "actual" and "potential"

This definite information might well suffice in the circumstances were it not for the fact that the intensity of urinary acidity is, among several, the most immediate indication of the magnitude of a process which constitutes one of the fundamental regulating activities of the organism the carefully balanced excretion of acid whereby the neutrality of the body is preserved[3] Accordingly precise and numerous data of urinary acidity, both normally and pathologically, appear to be desirable Fortunately the researches of Sorensen make this task an easy one His researches have made possible the convenient use of a considerable variety of indicators[4] and provided ingenious and convenient refinements

[1] Henry P Walcott Fellow in Clinical Medicine, Harvard Medical School

[2] *Pfluger's Archiv*, lxxxvi, p 586, 1901, *Beitr z chem Physiol*, iii, p 525, 1903, *Biochem Zeitschr*, xxiv, p 40 1910

[3] L J Henderson this *Journal*, ix, p 403, 1911

[4] See numerous papers in *Comptes rendus des travaux du Laboratoire de Carlsberg*

which enhance their accuracy Our investigations, recorded in this paper, yield a confirmation, if such be necessary, of the reliability of these methods

We have as before estimated the urinary reaction after the addition of indicators by matching colors of urinary samples with those of standard solutions of known reaction The standard solutions were made up as follows

TABLE I

NO	NaH_2PO_4	Na_2HPO_4	$\overset{+}{H}$	INDICATOR	
1		0 1000N	9 27	Phenolphthalein	
2	0 0001N	0 0480N	8 7		
3	0 0001N	0 0120N	8 0	Neutral red	Sodium alizarine sulphonate
4	0 0166N	0 0833N	7 48		
5	0 0010N	0 0060N	7 38		
6	0 0010N	0 0023N	6 90		
	CH_3COOH	CH_3COONa			
7	0 0009N	0 0920N	6 70	p-Nitrophenol	
8	0 0023N	0 0920N	6 30		
9	0 0046N	0 0920N	6 00		
10	0 0092N	0 0920N	5 70		
11	0 0230N	0 0920N	5 30	Methyl red	
12	0 0460N	0 0920N	4 90		
13	0 0920N	0 0920N	4 70		

In recording hydrogen ion concentration it appears to be both rational and convenient in the present case to use logarithmic notation as employed by Sorensen and others, rather than to record the actual concentrations, because the significant variation is in the logarithm of the numbers which represent the quantity $(\overset{+}{H})$ All such logarithms are of course negative and for convenience the minus sign is omitted The following table presents the conversion of the logarithmic notation into actual concentrations of ionized hydrogen

Our first care was to make a more detailed study of the effect of dilution of the urine upon its reaction and to see if a variety of indicators yielded concordant results It soon appeared that the reaction of undiluted urine could be readily estimated by employing colored standard solutions (Sorensen's method) The varying

TABLE II

Log	$\overset{+}{H}$	Log	$\overset{+}{H}$
4 6	250 × 10^{-7}	6 4	4 0 × 10^{-7}
4 8	160 × 10^{-7}	6 6	2 5 × 10^{-7}
5 0	100 × 10^{-7}	6 8	1 6 × 10^{-7}
5 2	63 × 10^{-7}	7 0	1 0 × 10^{-7}
5 4	40 × 10^{-7}	7 2	0 63 × 10^{-7}
5 6	25 × 10^{-7}	7 4	0 40 × 10^{-7}
5 8	16 × 10^{-7}	7 6	0 25 × 10^{-7}
6 0	10 × 10^{-7}	7 8	0 16 × 10^{-7}
6 2	6 3 × 10^{-7}	8 0	0 10 × 10^{-7}

colors of urine are as a rule easily reproduced in standard solutions with all desirable accuracy by the addition of *p*-nitrophenol, methyl orange, alizarine sulphonate or bismark brown Thereafter the substance which is to be used as indicator may be added in equal concentrations to urine and colored standard solutions, and that one of the latter which still corresponds to the urine in color may be selected

After the reaction of urine has been estimated in this manner with whatever may be convenient, it is an easy task to determine the effect of dilutions upon the reaction by diluting both urine and standard solution equally The following table presents a fair sample of our data of the reaction of diluted and undiluted urine

TABLE III

Indicator	Reaction		Indicator	Reaction	
	Undiluted	Diluted		Undiluted	Diluted
Para-nitrophenol	5 40	5 40	Sodium alizarine sulphonate	5 00	5 00
	6 70	6 70		5 00	5 00
	5 52	5 52		6 82	6 82
	5 82	5 82		7 10	7 10
Methyl red	5 00	5 00		7 22	7 22
	5 00	5 22		7 10	7 10
	4 82	5 00	Methyl red	6 82	6 82
	5 12	5 30		7 10	7 10
	4 70	4 82		7 22	7 22
				7 10	7 10

synthesis of tissue compounds is the only explanation generally held and taught today to account for the building up of protein, fats and carbohydrates

The work upon the synthesis of proteins *in vitro* through the agency of proteolytic enzymes, has not proven adequate to the task as yet, but the complexity of the reactions involved and the lack of those modifying influences which undoubtedly exist in the living cell are sufficient to account for the failures up to the present time But while no one has succeeded in building up a typical native protein, great success has attended the efforts toward the synthesizing of carbohydrates and fats The work of Hamsick[7] in particular may be cited, for he has succeeded in demonstrating a 30 per cent synthesis of triolein from its components He has further shown that the presence of bile salts is important in facilitating the synthetic reaction just as the hydrolytic It is not necessary to multiply examples of enzyme syntheses *in vitro,* since the fact is too well established to need further proof Attention should be called in passing, however, to the conditions under which successful reversions take place The absence of water from the reacting mixture is a prime necessity Croft Hill[8] used a 40 per cent dextrose solution in his synthesis of isomaltose—a concentration far above anything found in vegetable sap, so far as we know Hamsick used pure glycerine, oleic acid and dry pancreas powder in accomplishing his notable syntheses The reason for the small yield of ethyl butyrate obtained by Kastle and Loevenhart was undoubtedly the dilution of the reacting mixture A small active mass of water is as important in syntheses catalyzed by enzymes as in those catalyzed by mineral acids for example Concentrated sulphuric acid, pure fatty acids and alcohols are commonly used in laboratory ester syntheses, because under these conditions the mass of water is kept at a minimum and the hydrolytic reaction is correspondingly small It is well known that a mixture of ester, water and mineral acid tends toward hydrolysis rather than toward the reverse reaction so long as the water is above a certain proportion It has been shown[9] similarly that pancreatic lipase tends toward the complete hydrolysis of

[7] Hamsick *loc cit*

[8] Croft Hill *loc cit*

[9] Bradley this *Journal,* viii, p 251, 1910

triolein if water is present to the extent of 50 per cent of the reacting mixture, and the more water present the more rapid is the hydrolysis If less than 50 per cent of water is present equilibrium is reached before hydrolysis is complete It requires, however, a relatively small amount of water to secure a relatively large degree of hydrolysis and conversely a high degree of desiccation is necessary to produce noteworthy syntheses This fact, however, does not exclude the possibility of enzyme syntheses in cells where the total water content approximates 80 per cent, since there is abundant evidence of the fact that concentrations in localized portions of the cell may be far greater than in other adjacent portions The probable foam-structure of cytoplasm, the adsorption phenomena at the boundaries of different phases, gives us a mechanism which appears adequate to explain why at one point in a cell fat may be synthesized from its components, while at another point it may be undergoing hydrolysis at the same time Such data as have been presented by Macallum on the localization of potassium and other salts in growing structures, may be cited as proof of the possibility of attaining high concentrations of soluble and diffusible substances in sharply localized areas of the cell The familiar formation of excretory vacuoles in the unicellular organisms, where water charged with products of metabolism collects and grows, moves to the periphery of the cell and discharges into the surrounding medium, is a striking example of this phenomenon

So far therefore it seems quite possible to visualize in a crude way a mechanism by which syntheses by means of enzymes can take place in cells whose water content as a whole is too high to admit of this result once the cell structure is lost or disturbed The theory requires a complexity of structure and reaction which is abundantly borne out by other facts The theory has, however, never been given that close scrutiny which so broad a working hypothesis deserves Very little has been done to establish it beyond proving the true catalytic nature of enzyme reactions It has therefore seemed advisable to secure data upon this important phase of metabolism, confirming or disproving the theory as the case may be At the outset it has been evident that the methods of actually scrutinizing tissue syntheses are difficult and uncertain of interpretation, and that no single reaction or series

at Woods Hole in 1911 Lipase was estimated in the usual way by making up a definite weight of tissue "Brei" to a standard volume, adding a soluble ester, ethyl butyrate, and titrating the acidity developed After a period of three days the lipase reaction has usually reached equilibrium, so that the acidity at the end of that time represents roughly the amount of lipase present Fat was extracted from another weighed portion of the fresh tissue by boiling alcohol and ether The extracts dried at 105° were reextracted with ether, evaporated in a weighed capsule and dried to constant weight The results from this preliminary survey are not considered accurate, but they are sufficiently so to indicate that a correlation between lipase and fat content in the tissues examined does not exist Some of the tissues richest in fat are poorest in lipase, and *vice versa* The following table contains a few of these results

TABLE I

No	Tissue	Fat from 5 grams brei	$\frac{N}{10}$ acidity developed by 5 grams "brei" in 100 cc water 2 cc ethyl butyrate
		grams	*cc*
1	Shark liver	2 34	0 30
2	Dog fish liver	2 28	0 50
3	Dog fish liver	2 19	1 40
4	Shark liver	2 16	0 20
5	Shark liver	1 39	0 40
6	Dog fish liver	1 15	0 90
7	Flounder liver	1 09	2 00
8	Squeteague liver	0 84	0 85
9	Limulus liver	0 16	0 70
10	Shark sperm	0 10	0 40
11	Shark kidney	0 06	0 30
12	Shark pancreas	0 03	0 60
13	Limulus blood	0 02	0 30

It will be seen that while the lipase content of shark liver and Limulus blood is exactly the same, the fat of the former is one hundred times greater than that of the latter Again, No 6, dog fish liver, contains one-half the fat that shark liver, No 1, contains but its lipase number is three times as great Flounder liver contains less than one-half the fat of shark liver but seven times the amount of lipase

A comparison of such figures as those in Table I is not however satisfactory The lipase is presumably associated with the protoplasmic portion of the cell contents—the dry, fat-free proteins A comparison of shark liver, which is about 50 per cent fat, with other tissues, which contain little or no fat, on a basis of fresh tissue weight is obviously incorrect The real protoplasmic basis of fresh shark liver must be much less than that of mammalian liver, for example, and a comparison of the lipolytic activity of the two would appear to make the mammalian liver tissue more active than it actually is Furthermore the introduction of the insoluble ester triolein along with the shark liver "Brei" must introduce a further error of comparison the extent of which we do not know In comparing aliquots titrated with $\frac{N}{10}$ NaOH and $\frac{N}{10}$ KOH (alcoholic), it was found that the acidity in the alcoholic titration was frequently nearly double that of the water solution Evidently the lipase acts on both the soluble and the insoluble ester at the same time and at approximately the same rate If there were no fat introduced along with the "Brei" it would seem reasonable to assume that the ethyl butyrate would be hydrolyzed more rapidly It is fair to state however that, after making all possible allowance for this latter factor, the general results would remain quite unmodified

A second, more accurate investigation was made, with a view to determining what corrections should be made on the basis of protein or protoplasmic contents of the tissues examined[11] Fat was estimated, the dry weight of the tissues, and the lipase activity Calculating the dry, fat-free weight of the tissues it is seen that a wide range exists—from 4 to 40 per cent We may assume that 20–25 per cent represents about the average Then a tissue like shark liver which may contain as little as 4 per cent fat-free substance, represents only about one-fifth or one-sixth of the amount of active tissue found, for example, in porpoise liver As has been shown, the law of Schutz holds approximately through a considerable range of enzyme concentration the height of digestion varies with the square root of the enzyme, when equilibrium is allowed to be attained Accordingly four times the enzyme present should double the acidity developed at equilibrium

[11] I wish to acknowledge the valuable assistance of Mr Eugene Kellersberger in securing these data

TABLE II

NO	SPECIES	TISSUE	FAT	RESIDUE	PROTEIN	$\frac{N}{10}$ ACID PER 5 CC ALIQUOT	CORRECTION FACTOR	CORRECTED ACIDITY NUMBER
			per cent	*per cent*	*per cent*	*cc*		
1	Porpoise	liver	5 06	26 44	21 38	9 20		9 20
2	Tautaug	liver	49 20			4 60		4 60
3	Pogy	liver	13 37			4 00		4 00
4	Dog fish	liver	39 35	52 58	13 23	1 80	2	3 60
5	Dog fish	liver	34 69			3 15		3 15
6	Shark	liver	63 14	66 52	3 38	0 50	7	3 50
7	Shark	liver	62 58	66 52	3 94	0 40	6	2 40
8	Skate	liver	12 50			2 30		2 30
9	Skate	liver	16 45			2 10		2 10
10	Shark	liver	45 49	56 45	10 96	0 65	2	1 30
11	Shark	liver	45 59	56 78	11 19	0 65	2	1 30
12	Dog fish	liver	29 72	50 56	20 84	1 25		1 25
13	Shark	liver	58 12	65 86	7 74	0 40	3	1 20
14	Shark	liver	37 70	49 88	12 18	0 60	2	1 20
15	Dog fish	liver	17 24			1 15		1 15
16	Shark	liver	51 58	60 70	9 12	0 50	2	1 00
17	Sycotypus	liver	7 91	32 97	25 06	1 70		1 70
18	Sycotypus	liver	5 23	29 97	24 74	1 00		1 00
19	Star fish	dig gland	15 78	36 03	20 25	1 00		1 00
20	Limulus	dig gland	2 83	13 48	10 65	0 30	2	0 60
21	Limulus	muscle	0 79	21 09	20 30	1 40		1 40
22	Tautaug	muscle	0 90	20 42	19 52	1 20		1 20
23	Tautaug	muscle	0 90			0 90		0 90
24	Sycotypus	radula mus	1 62			0 65		0 65
25	Sycotypus	pedal mus	0 95	26 89	26 00	0 50		0 50
26	Skate	muscle	0 60			0 50		0 50
27	Limulus	muscle	0 76	13 85	13 09	0 50	2	1 00
28	Skate	muscle	0 78	31 71	20 29	0 40		0 40
29	Sycotypus	pedal mus	0 78	24 67	23 89	0 30		0 30
30	Pogy	red mus	8 80	28 13	19 33	0 30		0 30
31	Dog fish	muscle	1 25			0 25		0 25
32	Pogy	white mus	1 33	22 00	20 67	0 20		0 20
33	Dog fish	muscle	0 70	21 38	20 68	0 15		0 15
34	Dog fish	white mus	1 01	23 80	22 79	0 00		0 00
35	Dog fish	red mus	2 65			0 20		0 20
36	Shark	muscle	0 74			0 00		0 00
37	Shark	heart mus	2 93			0 25		0 25

TABLE II—Continued

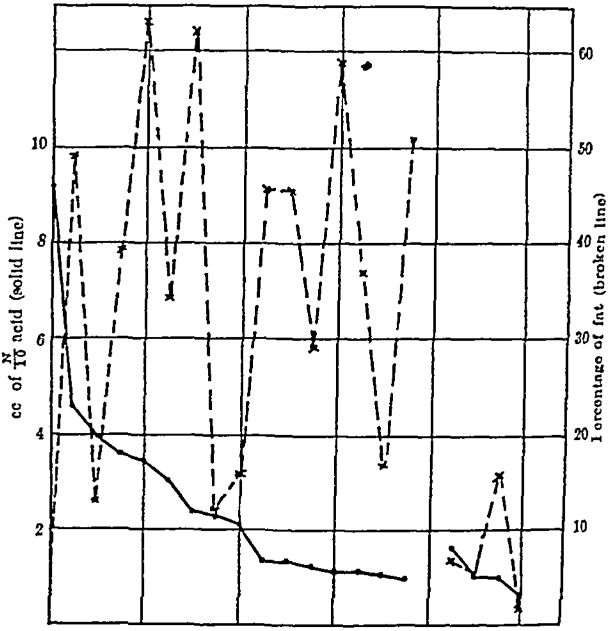
NO	SPECIES	TISSUE	FAT	RESIDUE	PROTEIN	$\frac{N}{10}$ ACID PER 5 cc ALIQUOT	CORRECTION FACTOR	CORRECTED ACIDITY NUMBER
			per cent	*per cent*	*per cent*	*cc*		
38	Limulus	eggs	8 29	49 00	40 71	1 50	½	0 75
39	Limulus	eggs	8 90	48 95	40 05	1 30	½	0 65
40	Skate	eggs	6 17	33 15	26 92	0 70		0 70
41	Star fish	eggs	3 38	11 49	8 11	0 30	3	0 90
42	Skate	ovaries	3 92			1 60		1 60
43	Skate	album gland	2 59	34 58	31 99	1 00		1 00
44	Shark	testis	3 42			4 10		4 10
45	Tautaug	testis	2 92			0 90		0 90
46	Shark	spleen	2 31			2 30		2 30
47	Shark	spiral valve	2 40			0 90		0 90
48	Limulus	blood	0 00			0 90		0 90
49	Limulus	blood	0 01			0 45		0 45
50	Shark	blood	0 24			0 15		0 15
51	Sycotypus	blood	0 31			0 00		0 00
52	Sycotypus	blood	0 02			0 00		0 00

Since this proportionality is not a strict one and only holds through a limited range of variation of the enzyme, we have decided to give every possible advantage to the tissues, poor in protoplasmic residue, in the tabulated comparisons We have therefore assumed a direct or linear relation between enzyme and acid developed As will be seen in the preceding table, even this excessive correction allowance fails to account for the differences which we find between homologous organs of different species, nor does it greatly lessen the striking lack of correlation between fat and lipase

Graphs have been prepared to show the relationship between lipase and fat, segregating the figures for muscle, liver, digestive glands, eggs, etc The lack of parallelism is quite evident

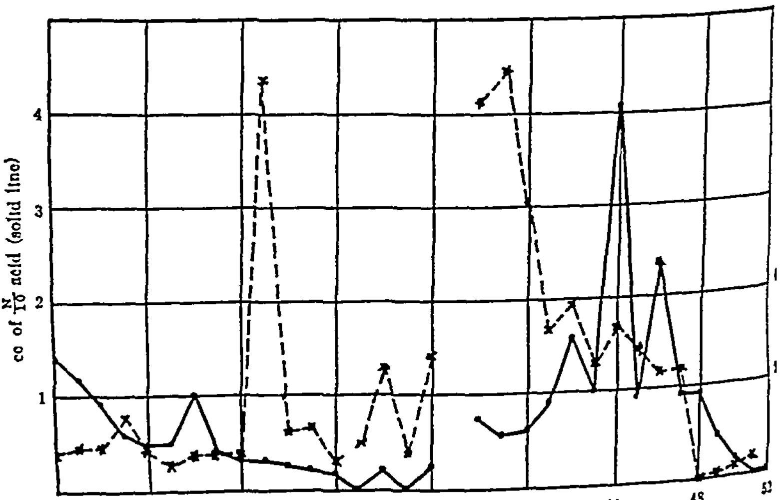
It may be suggested that the comparison of tissues from widely different species of animals is hardly a fair one The function of the liver of the teleost must be very different quantitatively at least from that of the mammal Shark and dog fish livers are always found rich in fat, while mammalian liver is uniformly poor But a series of figures limited to sharks and dog fishes still shows no parallelism between the fat and lipase

LIVER TISSUE



No 1 5 9 13 16 17 20

MUSCLE MISCELLANEOUS TISSUE



No 21 25 29 33 37 38 40 44 48 52

In the following table are presented some miscellaneous figures,[12] which cannot be strictly compared with those of preceding tables since the digestions were carried on at 37° instead of 25°

TABLE III

SPECIES	TISSUE	$\frac{N}{10}$ ACIDITY PER 5 CC ALIQUOT	FAT
		cc	*per cent*
Cat	liver	7 80	7 01
Rabbit	liver	6 40	4 00
Dog	liver	6 20	3 23
Perch	liver	5 00	4 52
Shad	liver	3 40	13 00
Calf	lung	4 80	2 44
Beef	kidney	4 00	2 17
Calf	heart muscle	2 90	2 27
Shad	spermatoid	2 70	2 40
Cow	mammary (active)	1 90	11 00
Goat	mammary (active)	1 50	4 30
Calf	brain	1 10	4 72
Goat	blood	0 80	0 12
Dog	thoracic lymph	0 07	0 78

Attention should be called to the interesting observation, which I have frequently confirmed, that active mammary tissue is less rich in lipase than mammalian liver, and even than heart muscle, beef kidney and calf lung, although it is richer in fat than these tissues and is daily secreting very large amounts of fat in the milk, in addition Instead of being unusually rich in lipase on account of its active secretion of fat, it is found to be about on a par with such other tissues as fish ovaries and testes, spleen, brain and other gland structures of mammals and invertebrates Compared with a large number of tissues active mammary gland is not a tissue rich in lipase, it is only twice as active as blood itself The fact that active mammary tissue is richer in lipase than inactive, as was pointed out by Loevenhart, is probably due in large measure to its hyperplastic condition during lactation It is certain that the secreting epithelial cells are far more abundant in the active gland than in the resting one, so that a given weight of active gland tissue must contain a far greater proportion of gland cells and

[12] Data collected by Clarence Schuldt for graduation thesis

correspondingly less connective tissue than the same weight of resting mammary Not knowing the relative proportion of epithelial cells in the two types of tissues it is impossible to estimate how much of the increased lipolytic activity of the secreting gland is due to increased metabolic activity of the cells themselves, and how much to their numerical increase

CONCLUSIONS

1 No broad correlation exists between the fat and lipase content of tissues

2 Homologous organs in allied species, such as teleost livers, fish muscles, etc , show no parallelism between fat and enzyme

3 Some of the most active fat-producing tissues are relatively poorer in lipase than many other tissues which never normally contain or produce more than a small percentage of fat Active mammary tissue affords the most striking example of this when compared with lung, kidney and muscle tissues

4 Quantitative comparison of fat and lipase in animal tissues gives no positive evidence in support of the theory of enzyme synthesis

THE PROBLEM OF ENZYME SYNTHESIS II

DIASTASE AND GLYCOGEN OF ANIMAL TISSUES

By H C BRADLEY AND E KELLERSBERGER

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(Received for publication, November 2, 1912)

In a recent paper by Hugh MacLean,[1] diastatic activity of mammalian tissues was reported to have the following order kidney, lungs, liver, heart, stomach and bladder and skeletal muscles There were individual and species variations found, but the average diastatic activity was in the order named It is interesting to note that kidney and lung tissues which are most active, are seldom found to contain more than traces of glycogen, if any Muscles, which are least active diastatically, frequently are quite rich in glycogen and normally contain some of that carbohydrate MacLean pointed out the lack of correlation between glycogen storage and diastatic activity in the tissues examined

We have extended this field of inquiry to include a number of lower forms known to be rich in glycogen For example the muscle tissues of molluscs are usually very rich in glycogen, so much so indeed that it makes one of the best sources for the preparation of large amounts of this substance Are such muscles proportionately rich in diastase?

MacLean's method of preparing the tissues for examination was followed The living tissue was ground fine into a large excess of alcohol and kept thoroughly mixed The alcohol was changed several times in twenty-four hours, pressing the pulp dry each time in a meat press At the end of this period the tissue was washed quickly with ether and gasoline to remove alcohol and fats, pressed dry and spread out in thin layers on filter paper in a current of air The dry pulp was re-ground and sieved Two grams of this powder, 25 cc of a stock starch solution used throughout the experiments and a little toluene were made up to 100 cc The time

[1] MacLean *Biochem Journ* , iv, p 467 1909

required for the disappearance of the starch and the erythrodextrin reactions was used as the indication of the activity of the diastase present We have also used glycogen solutions for digestion but have found such complete parallelism between the starch and glycogen digestions that the former has been adopted in our routine examinations It is believed that for comparative purposes, such as these experiments require, the change to achroödextrin, as indicated by the iodine reaction, is as valuable as a quantitative determination of sugar at the end of a fixed time

Glycogen was estimated in a weighed sample of the tissue powder by the Pfluger method, boiling with strong alkali for two hours, precipitating with alcohol, redissolving and reprecipitating several times, and finally weighing the purified substance dried to constant weight Where traces only could be found they were used for qualitative reactions for the identification of the substance as glycogen In a few cases where only a limited amount of the tissue could be obtained the fresh "Brei" was used for the detection of diastase

NO	SPECIES	TISSUE	DRY WEIGHT	TIME	GLYCOGEN	REMARKS
			grams		*per cent*	
1	Pecten	liver	2	37 min	trace?	
2	Sycotypus	liver	2	47 min	1 15	
3	Rat	liver	2	60 min	trace	
4	Limulus	liver	2	90 min	none	
5	Sand shark	liver	2	240 min	none	
6	Sand shark	liver	2	480 min	none	
7	Limulus	eggs	2	570 min	2 57	
8	Limulus	muscle	2	2½ day	trace	
9	Dog fish	liver	2	5 days	1 35	
10	Pecten	muscle	2	5 days?	4 62	erythro dex *
11	Squid	muscle	2	5 days?	none	
12	Dog fish	muscle	2	6 days?	0 20	
13	Sand shark	muscle	2	7 days?	none	
14	Squeteague	muscle	2	7 days?	trace	
15	Lobster	muscle	2	7 days?	none	
16	Sand shark	muscle	2	8 days?	none	
17	Sycotypus	muscle	2	8 days?	3 78	blue-purple
18	Swordfish	muscle	2	?	none	

* Where a question mark appears it indicates that no sure indication of digestion could be detected at the end of the time given to that experiment In the two cases noted Nos 10 and 17 there was some slight indication of change in the starch solution though in 17 it was not clear whether it was due to digestion of starch or not

NO	SPECIES	TISSUE	FRESH WEIGHT	TIME	GLYCOGEN	REMARKS
			grams			
19	Sycotypus	muscle	10	8 days ?	0 68	
20	Shark	muscle	10	3 days	trace	
21	Sycotypus	blood	75	3 days	none	
22	Squid	liver	3	5 days	none	
23	Squid	blood	25	3 days	none	
24	Squeteague	liver	3	3 hours	trace	
25	Lobster	liver	25	3 hours	trace	
26	Lobster	blood	25	?	none	
27	Sycotypus	liver	10	3 hours	none	
28	Shark	liver	10	5 hours	none	

An analysis of the results shows in the first place that the treatment to which the tissues are subjected in drying and rendering them fat-free, does not greatly alter the diastatic activity, compare 2 and 27, 4, 6 and 28 It is improbable therefore that, by this treatment, diastase has been destroyed in any of the tissues which fail to digest starch

In the second place, the most active tissues diastatically, Pecten, Sycotypus, rat, Limulus, and shark livers and Limulus eggs, do not all contain glycogen It is quite probable however that in periods of nutritional plenty these tissues might all contain glycogen, since with one exception they are livers or digestive glands which are believed to function like the liver in storing glycogen On the other hand, the least active tissues are the muscles With the exception of Limulus muscle (which contains no glycogen) these tissues are practically devoid of diastatic enzymes Yet Pecten and Sycotypus muscles are the richest tissues which we have analyzed for glycogen In the case of Pecten muscle there was an unmistakable change to erythrodextrin, but during the five-day digestion period there seemed no further change At the end of an eight-day period Sycotypus muscle showed no more change than expressed by a slight purple tint in the digestion mixture when treated with iodine, instead of the original clear blue A control of 10 grams of the perfectly fresh muscle "Brei" showed no digestion at the end of eight days Inasmuch as Sycotypus blood showed diastase, it is not surprising that the muscle sample 17 appeared to have a trace of activity Indeed it might be considered more surprising that the tissue showed so

little When the gasteropods were killed, however, the large blood spaces of the pedal muscle were cut in several directions to allow free bleeding, and the muscles themselves under this stimulus contract to the utmost, thus squeezing out practically all of the blood present What extremely slight digestion appeared to go on in 17 we may attribute to a trace of blood rather than to the muscle fibres themselves

It should be noted that our figures disprove the statement of Abderhalden,[2] that "With the gasteropods the liver is the only place in which glycogen is deposited to any extent, in the other organs the amount is hardly worthy of consideration" The gasteropod, *Sycotypus canaliculatus*, regularly contains large amounts of glycogen in the pedal muscle The specimens here examined had been kept in captivity for many weeks, long enough to remove most of the glycogen from the liver in several individuals examined and to have reduced the percentage present in the muscles Freshly dredged specimens in good condition will yield much more glycogen than the figures above would indicate The same is true of the muscles of many lamellibranchs, of which Pecten is an example

In confirmation of MacLean's work, we find that tissues rich in diastase may or may not contain glycogen, and, what is far more significant from the point of view of the enzyme-synthesis theory, tissues rich in glycogen may or may not contain diastase While it is easy to conceive of a tissue rich in an enzyme failing to build up the synthetic product of that enzyme's activity, it is not so easy to explain the absence of an enzyme from a tissue regularly building up a certain compound, if we attribute that synthesis to the enzyme At the same time the absence of the enzyme does not invalidate the general theory It is possible that the enzyme has disappeared after the synthesis, or that the diastase of the blood is capable of diffusing into the cells at certain times, or that in an animal whose whole metabolism is on so low a level, an amount of diastase too small to detect is still sufficient to synthesize and hydrolyze the glycogen stored in the pedal muscle It is not impossible of course that glycogen is synthesized in the active liver or "hepato-pancreas" and is transported thence to the muscle tissue in the blood The presence of glycogen in the latter might

[2] Abderhalden *Text-book of Physiological Chemistry*, p 46

be too slight also for detection at any given time There are many considerations which render the negative results, recorded above, equivocal rather than negative of interpretation It is clear however that the experiments do not add anything to substantiate the theory

THE PROBLEM OF ENZYME SYNTHESIS III

DIASTASE AND STARCH OF PLANT TISSUES

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(Received for publication, November 2, 1912)

It is well known that diastase is widely distributed in the active tissues of plants Just what its relative strength is in different tissues has been less carefully determined Furthermore the quantity of the enzyme is variable so that only very general statements can be made concerning its presence The season of the year, the condition of the plant and its stage of development, etc , all exercise an influence in determining the activity of the enzyme For this reason much of the data collected upon this question was unavailable for the purposes of this investigation, and a series of determinations was therefore made to determine whether a relationship exists in the plant tissues between starch and the enzyme which is believed to synthesize it from the more soluble carbohydrates A definite correlation between the enzyme diastase and the starch content would throw much light on the general question of enzyme synthesis The plant should be especially favorable material for this problem because of the relatively simple metabolism there found

The method used was that described in a previous paper for the detection and estimation of diastase in animal tissues The plant organs were not treated with alcohol and ether since few plant tissues contain appreciable amounts of fat Five grams of the finely pulped material were weighed out, 25 cc of the same starch solution added in all cases, toluene and water to make the volume 100 cc The iodine reaction was used as the test of digestion, and the time required to convert the starch solution to the achroodextrin stage indicated in a rough way the activity of the diastase present A number of tissues were assayed for their dry weight as a further check on the interpretation of the figures of d n

A number of objections may be urged at the outset First The method is not a quantitative one, the end point with iodine is not sharp and a considerable error is thereby introduced Making all possible allowances for error in this direction does not however invalidate the general results—a given tissue is ten or a hundred times more active than another

Second Many tissues already contain starch in larger or smaller amounts, and the presence of this starch must inevitably alter the time required for digestion to the achromic point This error is in many cases a serious one In many others however the starch grains are so resistant to the diastase that filtering the samples taken for tests removes them, and the solution then represents the original starch solution added We have found a number of tissues where the reaction of the starch grains in suspension persisted hours or even days after the soluble starch solution had all been converted into dextrins which gave no iodine color reaction

Third Very noticeable differences exist between the action of diastases of different origins In some cases erythrodextrin is rapidly formed from the starch, and then persists for long periods of time In others no erythrodextrin reaction develops, the solution remains blue-reacting to the end, and merely decreases in intensity to the vanishing point In others the steps from starch to erythrodextrin to achroodextrin are well defined and follow each other in approximately the same time This difference is possibly an indication of a complex of enzymes in what is called "diastase," one factor of which hydrolyzes starch, another erythrodextrin, etc If, in a given tissue "Brei" the erythrodextrin-digesting enzyme is much more abundant than the starch-digesting, then the speed of the entire reaction will be expressive of the speed of that initial step of hydrolysis The reddish color at the same time will never be appreciable, since the erythrodextrin will be hydrolyzed as rapidly as formed In other cases, where the initial step is rapidly taken but the dextrinase is wanting, the mixture rapidly assumes the red-staining stage and then very slowly or never passes on to the achromic point

Razor sections of the organs were stained with iodine, and where this reaction was negative or equivocal, samples of the "Brei" were extracted with boiling water and the extract tested for starch

TABLE I

SPECIES	TISSUE	DRY WEIGHT	DIGESTION TIME	STARCH CONTENT
		per cent	*hours*	
Green pea	leaf		0 70	None
	pod and seeds	12 4	0 50	Small amount
	seeds, medium	24 6	0 50	Medium
	pods, medium	13 2	0 80	Small amt dextrin
	seeds, mature	80 0	1 00	Abundant
Lima bean	seed, medium	23 2	6 00	Abundant
	pod, medium		5 00	Small amt dextrin
	leaf	15 4	0 50	Small amt dextrin
Kidney	seed, medium	27 6	5 50	Medium
	pod, medium	14 8	5 50	Abundant dextrin
	leaf	18 0	0 25	Small amt dextrin
Kohlrabi	root		1 50	None
	leaf		1 50	None
Onion	root, young	13 4	?	None
	leaf, young	10 6	48 00	None
Bayberry	leaf		72 00	None
	berry, mature		72 00	None
Radish	root, mature	5 6	0 70	None
	leaf, mature	8 6	0 70	None
Carrot	root, medium	10 8	2 50	Abundant
	leaf, medium		22 50	None
Red beet	root, young		?	None
	leaf, young		1 25	None
	root, mature		14 00	None
	leaf, mature		6 00	None
Mangel	root, mature		?	None
	leaf, mature		7 00	None
Potato	root, medium		18 00	Some dextrin
	tuber, medium		17 00	Very abundant
	leaf, medium		0 75	None
Corn	seed, medium		7 50	Abundant
	husk, medium		6 00	None
	seed, mature		6 50	Abundant
	cob, mature		6 50	Small amount
	seed, young		5 00	Medium amount
	cob, young		6 50	Small amount
	husk, young		2 00	None
Marrow squash	seed, mature	25 2	1 25	Medium
	pulp, mature		?	Abundant
	pulp, young		48 00	Abundant
Cantaloupe	leaf, mature		0 50	None
	pulp, mature		72 00	None
	seed mature		?	Small amount

In the table the relative amounts of starch found are indicated In general it is a fact that leaves contain starch In some it is found always, in others only while photosynthesis is in rapid progress The fact that our plant tissues were usually secured early in the morning, before photosynthesis had gone on to any large extent, is probably the explanation of our failure to find starch in many of the green parts of plants During the night, the starch, in such parts as the leaves, usually disappears It is believed to be converted into sugar and removed by the sap to the organs of storage

About one hundred tissues were examined in this way The results are very divergent A few of the more striking figures are given in the table below The publication of the rest would not add materially to the solution of the present problem

The above typical results, together with the mass of data unpublished, shows the following conclusions are warranted

First Diastase is as a rule most abundant in the leaves, where starch is never stored permanently, but where it may be found in small amounts during photosynthesis Leaves of different species exhibit very marked differences in their diastase content The leaves of the bayberry, onion and leek contain so little of the enzyme as to be doubtful On the other hand the leaves of the legumes were among the most active tissues examined It is of interest to note that the leaves of the legumes were usually found to contain considerable starch or a dextrin-like compound, though there were several exceptions noted

Second No general deductions can be drawn relating the diastatic activity of a tissue with its starch-storing function We find plants like the beets and mangels, which store no starch in the root but instead store sugar, having a highly active leaf and no diastase in the root Other plants like the radish and rutabaga and kohlrabi, which also store sugar instead of starch in the root, are diastatically active in both leaf and root The radish root is one of the most active tissues we have found, despite the fact that it contains only 5 per cent solid material, and yet no trace of starch could be found in the many samples examined Other plants, like carrot, parsley and parsnip, lay up starch in the root which is diastatically active—in these cases more active than the leaf The potato tuber is particularly rich in starch but rather poor in diastase

In a very few cases tissues which were found to contain starch were so slightly active diastatically that they appear doubtful The pulp of the marrow squash and the seed of the cantaloupe eventually carried digestion to the erythro stage, at which point it appeared to stop completely Fresh mushroom tissues, after five days' digestion, showed no definite change of color Curiously enough, these same tissues ground up in alcohol, washed with ether and dried, developed considerable diastatic activity Did the treatment activate a proenzyme? If so, and the evidence of the single series of observations suggests this, why should the rapidly growing fresh tissues, rich in the glycogen-like carbohydrate of the mushroom, show no diastase? In the developing mushroom, which frequently grows several inches in a few hours, and in which the glycogen transfer must be unusually rapid, one would expect to find diastase in abundance if that enzyme is necessary for synthesis

With the exception of these doubtful cases just mentioned the results of our series offer more favorable material to interpret from the standpoint of enzyme synthesis than any other investigation which we have made The fact that practically all of the starch-storing tissues of plants are found to contain diastase, and that during the developmental stages as well as during sprouting, lends considerable support to the view that the enzyme is responsible for the synthesis of the polysaccharide from the sugar of the sap It must not be lost sight of however that even these favorable results are by no means decisive, the data may be interpreted in a different way, and there is nothing in the experiments which is crucial Furthermore if any of the doubtful cases cited should on further work prove to be actually negative, the value of the positive cases as favorable evidence for the theory would be entirely lost At present, however, it appears to us that the strongest evidence for the theory is to be found in the relation of starch and diastase in the organs of plants There are many tissues rich in diastase which never develop starch, but on the other hand there are no tissues which contain starch of which we can say that they are absolutely devoid of diastase What the function of the diastase of tissues which never contain starch is, we have not even conjectured, but it seems quite possible, from the data, that those tissues which develop starch as a store of reserve material may syr the starch through the agency of dinstase

THE PROBLEM OF ENZYME SYNTHESIS IV

LACTASE OF THE MAMMARY GLAND

By H C BRADLEY

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(Received for publication, November 11, 1912)

In the preceding papers reporting investigations into the theory of enzyme syntheses in tissues, the results have been equivocal and difficult of interpretation They have neither confirmed nor disproved the general proposition that enzymes assist in the synthetic production of compounds which they also hydrolyze It is desirable therefore to secure some more crucial test of the theory An organ or tissue whose chief function is synthetic and the products of whose synthesis are somewhat specific would make an ideal tissue to examine from this point of view The active mammary gland is perhaps the best example of this sort of organ It produces considerable amounts of protein, carbohydrate and fat, and the protein and carbohydrate are quite specific Neither casein nor lactose are found elsewhere than in milk, and the lactose requires a specific enzyme to hydrolyze it The number of tissues which contain this enzyme are moreover very limited As Plimmer[1] has shown, intestinal mucosa and the pancreas of suckling mammals regularly contain the enzyme, while it is wanting in the adult pancreas and in most adult intestines Its presence in significant amounts in the active mammary would therefore go far toward proving the theory under investigation

As a test of the theory we have therefore proposed the presence or absence of lactase in the active mammary cell The hydrolytic action of the enzyme is much more easily demonstrated than its synthetic action, so that the failure of Porcher[2] to get synthesis of lactose from a mixture of gland "Brei," dextrose and galactose,

[1] Plimmer *Journ of Physiol* , xxxiv, p 93, xxxv, p 20, 1906

[2] Porcher *Arch internat Physiol* , xxiii, p 356, 1909

is not conclusive His results merely indicate that under the conditions of dilution, etc, which obtained in his experiments reversion did not take place Dilution may have prevented Porcher from finding a synthetic effect, but dilution should only facilitate the hydrolytic reaction of the enzyme

Active mammary glands were therefore obtained from animals suckling their young and at the height of lactation Samples of the milk were secured in most instances and the presence of lactose demonstrated, as a preliminary precaution The glands were ground to a fine "Brei" and diluted with a known proportion of water and were either allowed to autolyze over night under toluene or used immediately The autolyzed and diluted "Brei" was strained free from connective tissue shreds and a known amount of the mixture added to a solution of Kahlbaum's C P lactose A control digestion was checked at once by the addition of mercuric nitrate, prepared according to the method of Patein and Dufau,[3] or by boiling and then adding the mercuric nitrate Other samples were allowed to digest from two to seven days under toluene at 37° At the end of this period the proteins were removed by mercuric nitrate, the large excess of mercury precipitated in an aliquot of the protein-free filtrate by NaOH, and H_2S run in to precipitate the remainder Excess of sulphide was then removed by $CuSO_4$ and the solution made up to a definite volume and filtered Sugar was determined in aliquots of this filtrate by the Allihn gravimetric method Another aliquot of the sugar solution was then completely hydrolyzed by boiling with H_2SO_4 for one hour, neutralized, made up to known volume and analyzed for sugar Thus the reducing power of each solution was checked by its reducing power after hydrolysis—an extremely important point in the work, since it enables one to determine whether there has been destruction of either dextrose or lactose during the digestion period The mere fact that the reducing power of the digest does not alter during a seven-day period is not of itself sufficient to prove the absence of lactase, since a concomitant destruction of sugar may have balanced the rate of hydrolysis Nor would an increased reducing power of the solution alone be a proof of the hydrolysis of lactose, since liberation of a reducing substance in the

[3] Patein and Dufau *Journ de pharm et de chim*, xv, p 221, 1902

gland "Brei" itself is quite possible The results of Plimmer may be subjected to valid criticism on this very ground, since increased reducing power of the digest was taken as proof of lactase, without determining also the amount of lactose remaining at the end of the digestion period As a matter of fact we have found some indications of both destruction of dextrose and of the liberation of reducing disaccharides in certain experiments The check has a further value In the precipitation of the proteins by the mercuric nitrate method the character of the precipitate is frequently different in different digestion flasks Presumably too there is a difference in the amount of sugar absorbed by such precipitates, so that several digestions made up with scrupulous attention to exact duplication of the amounts of sugar and "Brei" present, will frequently show appreciable differences in the amounts of sugar present in the final filtrates Such differences must be assumed therefore in the interpretation of results as experimental errors inherent in the process, and variations within such limits cannot be considered evidence of hydrolysis If lactase is present at all, however, a digestion of seven days should show changes far beyond these experimental errors, and give unequivocal evidence of hydrolysis

In carrying out the details of this work we have followed the procedure described by Plimmer as closely as possible in order that our results might be comparable with his, and that some judgment might be made as to the amount of lactase present in the mammary gland compared with the tissues examined by him

EXPERIMENT I *October, 1911* Cat with four kittens, toward the close of lactation but still nursing the kittens The milk contained a reducing sugar The glands, dissected free from fat and connective tissue, weighed 35 grams They were reduced to a fine "Brei" with the addition of toluene water and allowed to incubate at 37° for twenty-four hours The "Brei" was strained, the residue washed and the mixture made up to 250 cc with toluene water Four digests were set up

I 50 cc "Brei," 100 cc 5 per cent lactose solution incubated 24 hours
II 50 cc "Brei," 100 cc 5 per cent lactose solution incubated 48 hours
III 50 cc "Brei," 100 cc 5 per cent lactose solution incubated 72 hours
IV 50 cc "Brei," 100 cc 5 per cent lactose solution boiled at once as control

At the end of each digestion period 10 cc of mercuric nitrate solution were added, the mixture allowed to settle in the cold over night and filtered One hundred and twenty cubic centimeters of the filtrate were made exactly neutral to litmus with concentrated NaOH and sufficient water added to make the total volume 125 cc The precipitated mercuric hydroxide was then filtered, and H_2S passed into 100 cc of the filtrate till the slight excess of mercury was removed The excess of H_2S was precipitated by the addition of copper sulphate and the mixture made up to exactly 250 cc and filtered Sugar was determined in 25 cc aliquots of this filtrate, which contains a very slight excess of the copper salt insufficient to exercise any appreciable effect upon the sugar determinations The cuprous oxide was filtered on a Gooch, washed repeatedly with hot water, alcohol and ether, and dried at 105° for an hour Duplicate results were obtained in each case

Fifty cubic centimeters of the final filtrate were hydrolyzed with the addition of 2 cc of concentrated sulphuric acid, boiled for one or more hours, neutralized and made up to 200 cc Sugar estimations on this showed the total available reducing sugar and thus the undigested lactose could be determined

The twenty-four and forty-eight-hour digests show a slight increase in reducing power, which is more than counterbalanced by a decreased total sugar Our only explanation of this is the possibility of some slight bacterial action unchecked by toluene The seventy-two-hour digest shows little deviation from the control

TABLE I

NO	HOURS	Cu_2O	AVERAGE	TOTAL AVAILABLE	RATIO
		gram			
1	0	0 4355			
		0 4354	0 4355	0 6178	1 418
2	24	0 4491			
		0 4470	0 4480	0 5704	1 273
3	48	0 4411			
		0 4405	0 4408	0 5832	1 323
4	72	0 4352			
		0 4345	0 4348	0 6000	1 379

Experiment II *October, 1911* Goat in active lactation, secreting about 400 cc of milk per day The milk contained

lactose One hundred and sixty grams of gland tissue were secured, ground fine, diluted and autolyzed over night Liver tissue from the s[illegible] animal was made up in the same way as a check

The results show no digestion of lactose Where liver tissue is substituted for mammary there is a distinct loss of sugar

TABLE II

NO	TIME	GLAND PRESENT	REDUCTION AVERAGE	TOTAL AVAILABLE	RATIO	TISSUE
	hours	*per cent*				
1	0	14	0 4357	0 6180	1 419	Mammary
2	96	14	0 4366	0 6200	1 420	Mammary
3	0	28	0 4577	0 6252	1 366	Mammary
4	96	28	0 5674*	0 7688	1 355	Mammary
5	0	14	0 4715	0 6450	1 378	Liver
6	96	14	0 4517	9 6152	1 362	Liver

* A stronger lactose solution was used in this digestion by mistake the ratio clearly shows however that no digestion has taken place

EXPERIMENT III *November, 1911* Rabbit, twenty-four hours after dropping her litter The glands were swollen and full of milk Forty-five grams were ground fine, diluted to 250 cc and autolyzed over night The strained mixture was made up with lactose as in experiment I

In Nos 2 and 3 there is no evidence of a change of any kind, in 4, which was made distinctly alkaline to litmus with bicarbonate, there was a slight loss of sugar as shown by both the digestion and inverted sample

TABLE III

NO	HOURS	GLAND PRESENT	REDUCTION AVERAGE	TOTAL AVAILABLE	RATIO	REMARKS
		per cent				
1	0	18	0 3936	0 5360	1 362	
2	72	18	0 3939	0 5400	1 371	
3	168	18	0 3928	0 5398	1 374	
4	72	18	0 3751	0 5276	1 406	Alkaline with $NaHCO_3$

EXPERIMENT IV *December 1911* Goat in the height of lactation The milk was shown to contain lactose Two hundred

and ninety-five grams of gland tissue were obtained A 10 per cent lactose solution was used

In this case there seems to have been a progressive liberation of sugar, more striking in the digestion to which blood had been added The necessity of making a determination of the total available carbohydrate is clearly brought out in this instance, since an examination of the amount of reduction produced by the digestions alone would seem to indicate hydrolysis The ratio shows however that such has not been the case

TABLE IV

NO	HOURS	GLAND PRESENT	REDUCTION AVERAGE	TOTAL AVAILABLE	RATIO	REMARKS
		per cent				
1	0	35	0 5450	0 8072	1 481	
2	120	35	0 5762	0 8144	1 413	
3	120	35	0 5918	0 8560	1 447	Blood present

EXPERIMENT V *February, 1912* Cow in the height of lactation, killed as a demonstration of the tuberculin reaction No macroscopic lesions were found in the mammary gland, which weighed over three kilos Five per cent lactose solutions were used in the digestion mixtures

Two and three were set up as duplicate digestions, the differences between them indicate the extent of divergence between such duplicates which must be taken into account in interpreting results In five, the "Brei" was allowed to autolyze for seven days, the lactose solution then added and action stopped at once with mercuric nitrate The series seems to show a small increase in the total sugar content as well as a larger increase in the reduction of the mixture before hydrolysis That this is not a digestion of lactose itself is shown by Nos 4 and 5 Comparing Nos 1 and 4 there is some indication of increased disaccharide content It may be suggested that a mother substance of lactose is present in the gland cells, as was thought by Porcher, from which lactose is split off during the digestion period Whatever the explanation, the fact that a disaccharide is produced during digestion from the gland cells, is of itself in direct opposition to the theory that lactase is responsible for the lactose of the milk

TABLE V

NO	HOURS	GLAND PRESENT	REDUCTION AVERAGE	TOTAL AVAILABLE	RATIO	REMARKS
		per cent				
1	0	35	0 4059	0 5738	1 414	
2	72	35	0 4033	0 5752	1 426	
3	72	35	0 4184	0 5804	1 387	
4	168	35	0 4220	0 6078	1 441	
5	168	35	0 4252	0 5800	1 364	Lactose added at end of digestion

EXPERIMENT VI The mammary of the same cow was kept frozen hard at a temperature of about $-20°$ for several days While thus frozen it was possible to produce an exceedingly fine gland-snow with an ice shave Another series of digestions were set up with this material, varying the reaction by the addition of bicarbonate sufficient to make the mixtures slightly alkaline to litmus The presence of alkali in the mixture leads to destruction of the sugar, though toluene was abundant in the mixtures and they were well shaken every twelve hours to insure saturation throughout the mixtures There was no evidence of putrefactive changes in any of the mixtures, though bacterial count was not made It is possible that in alkaline reacting "Breis" bacterial action was not wholly checked by the toluene, though this explanation of the destruction of sugar seems unlikely In all the digests acidity is developed presumably through the action of lipase on the butter fats present

TABLE VI

NO	HOURS	GLAND PRESENT	REDUCTION AVERAGE	TOTAL AVAILABLE	RATIO	REMARKS
		per cent				
1	0	35	0 3995	0 5772	1 445	
2	96	35	0 3541	0 5052	1 427	
3	168	35	0 2735	0 3982	1 456	Alkaline
4	168	35	0 3731	0 5220	1 402	
5	168	35	0 3201	0 4648	1 452	Alkaline

DISCUSSION

It is quite evident that under the conditions of these experiments no appreciable hydrolysis occurs Therefore no lactase is present, for our only criterion of an enzyme is the demonstration of its activity It is possible that a proenzyme normally present has not been activated, though conditions have been varied in the hope of producing such activation Blood of the animal has been added to the mixtures, to determine whether it might not carry some kinase to the gland Digestions have proceeded under neutral, slightly acid and slightly alkaline conditions Sugar has been added at once to the freshly prepared gland-brei, and to "Brei" which has autolyzed for twenty-four hours The result has been negative in each case In certain digestion mixtures there has been an increased reduction observed as digestion continued, which might have been mistaken for hydrolysis of lactose were it not for the control hydrolysis by acid In such cases the evidence points to the formation of a reducing disaccharide rather than to the conversion of disaccharide to monosaccharides of greater total reducing power It seems safe to assume that this disaccharide is lactose, developed from some mother substance already laid up in the gland cells at the time of death This mother substance may be analogous to glycogen, though the negative results of Porcher and other investigators makes this improbable, or it may be some more complex compound like a protein-lactose conjugation The very fact however that autolyzing mammary tissue develops a reducing disaccharide, presumably lactose, is itself strong evidence that lactose is not built up by the enzyme lactase in the active gland

There is further evidence that the production of lactose is not through the immediate agency of lactase The secretion of milk is a violently eruptive process in which cells are ruptured and their contents poured out into the ducts Following the manipulation of the teat by the suckling young, this eruptive secretion is set in motion by a reflex nervous mechanism During the periods between suckling, the milk precursors are stored in the mammary cells as is shown by the swelling of the whole gland If lactase were present at the moment of secretion it would certainly be swept out of the cells along with the milk constituents and would appear

in the secretion If it were present in the milk, dextrose and galactose would gradually appear in place of the lactose But dextrose is not found in normal fresh milk, nor on standing a few hours or days does it appear At the moment of secretion therefore we must conclude that the enzyme is not present It is possible that mammary lactase is an enzyme which is destroyed almost as rapidly as formed, but such a hypothesis is wholly without foundation in data thus far collected Lactase from other sources is apparently quite resistant

So far as this experiment goes, then, it gives no confirmation of the theory of enzyme syntheses On the contrary it is so thoroughly negative in its results and so crucial in character that it must cast some doubt upon the general hypothesis It appears justifiable to conclude that lactose of the milk is not synthesized through the agency of the enzyme lactase

THE ACTION OF YEAST ON YEAST NUCLEIC ACID

By SAMUEL AMBERG and WALTER JONES

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(Received for publication, November 12, 1912)

Upon a former occasion we showed that rabbit's serum brings about a depression of the optical rotation of yeast nucleic acid without setting free either phosphoric acid or purine bases and apparently without causing any deep-seated chemical alteration of the substance On the other hand thymus nucleic acid was found unchanged in any way by rabbit's serum since not even an alteration of the optical properties could be observed [1] In the meantime Levene and Jacobs[2] have been led to conclude that the two nucleic acids are quite dissimilar in their chemical constitution so that differences in their biological conduct are to be expected

We have recently had occasion to note a very marked difference in the behavior of these two nucleic acids towards various preparations of yeast, having uniformly found that yeast nucleic acid disappears, while under the same conditions thymus nucleic acid persists

Experiments were made with a number of preparations, especially brewer's yeast, compressed yeast and yeast powder which we prepared by the method of Lebedew,[3] but the results were always the same in that thymus nucleic acid was unchanged and could be precipitated by the addition of sulphuric acid while yeast nucleic acid disappeared and its decomposition products could be found Thus a 1 5 per cent solution of yeast nucleic acid was completely decomposed by compressed yeast extract in three days while a solution of thymus nucleic acid under the same conditions was apparently unchanged after digestion for nineteen days In an experiment with extract of yeast powder

[1] Amberg and Jones this *Journal*, x, p 81

[2] *Ibid* , xii, p 411

[3] *Zeitschr f physiol Chem* , lxviii, p 447

a 4 per cent solution of yeast nucleic acid was completely decomposed in three days while a 0 5 per cent solution of thymus nucleic acid was unaltered at the end of a month

The decomposition products of yeast nucleic acid obtained under the conditions stated are not without interest as will be seen from the following general summary of results

	COMPRESSED YEAST	YEAST POWDER
Autolysis	Adenine and xanthine	Adenine and guanine
With addition of yeast nucleic acid	Adenine and guanine	Adenine and guanosine

It will be observed first, that where one is dealing with an uninjured yeast preparation (as compressed yeast) and where the amount of nucleic acid to be decomposed is comparatively small (as in autolysis) the adenine group is set free but not deaminized while the guanine group is both liberated and deaminized This may be defined as the normal condition for yeast and is in accord with the older findings that yeast contains guanase but no adenase[4] But the occurrence of adenine and the failure of hypoxanthine among the products of auto-digestion of yeast are matters that are unique in nucleolysis There are two paths along which hypoxanthine may be formed from yeast nucleic acid the one by liberation and deaminization of adenine, a decomposition to which the presence of *adenase is indispensable, the other*, by the initial formation of adenosine which is transformed successively into hypoxanthosine and hypoxanthine, a succession of reactions which can occur in the absence of adenase

$$O = PO\,\boxed{C_5H_8O_4\ C_5H_2N_4(NH_2)} \longrightarrow \underset{\text{Adenosine}}{C_5H_9O_4\ C_5H_2N_4(NH_2)} \longrightarrow \underset{\text{Hypoxanthosine}}{C_5H_9O_4\ C_5H_2N_4(OH)}$$

HO

$$\downarrow$$

$$\longrightarrow \underset{\text{Adenine}}{C_5H_3N_4(NH_2)} \longrightarrow \underset{\text{Hypoxanthine}}{C_5H_3N_4(OH)}$$

Fragment of yeast nucleic acid

Most animal tissues do not contain any adenase so that one of these paths of hypoxanthine formation is out of question,

[4] Straughn and Jones this *Journal*, vi, p 245

nevertheless all such tissues bring about a formation of hypoxanthine from nucleic acid[5] and evidently along the second path under consideration But the yeast is characterized by its inability to bring about a deaminization of the adenine group (whether this be free or in combination) and therefore cannot produce hypoxanthine from any of its purine precursors It will be seen in the experimental part which follows that, no matter how the experiment is arranged adenine is *always* formed, hypoxanthine, *never*

In the second place, it will be noted, that yeast powder which was prepared by manipulation of yeast has lost the power of converting guanine into xanthine, which is to say, the ferment guanase, normally present in fresh yeast, has been destroyed We have had occasion to notice the easy destruction of guanase in other connections For instance, a dog's liver was perfused with large quantities of distilled water until all hemoglobin had been removed An extract of this bloodless liver was found free from guanase although a second dog's liver perfused with normal saline was found to possess the ferment unimpaired In both instances hypoxanthine was found but no adenine

Finally, it may be observed that the end products of the action of yeast on yeast nucleic acid depend upon the initial amount of nucleic acid to be decomposed—a curious phenomenon which was produced both by fresh yeast and by yeast powder Compressed yeast proceeds with a small amount of nucleic acid as far as xanthine but when the initial amount of nucleic acid is considerably increased, guanine is found as the end product Similarly, yeast powder can liberate guanine from a small amount of nucleic acid, while a larger amount of nucleic acid proceeds only as far as guanosine

The isolation of purine derivatives and phosphoric acid from digested yeast preparations is much more difficult than in the case when one is dealing with glandular extracts, so that severe losses often occur in the estimation of the products of digestion Nevertheless the results obtained are sufficient to establish the conclusions that have been stated

[5] Amberg and Jones *Zeitschr f physiol Chem*, lxxiii, p 407

EXPERIMENTAL PART

EXPERIMENT I 300 grams compressed yeast, 1000 cc water digestion, 19 days

685 cc for purine bases	*Without hydrolysis* gram
Adenine picrate	0 661
Xanthine	0 117
100 cc for phosphorus	
$Mg\ NH_4\ PO_4\ 6H\ O$	0 660

EXPERIMENT II 275 grams compressed yeast, 920 cc water, 15 grams yeast nucleic acid digestion, 19 days

400 cc for purine bases	*Without hydrolysis* gram	*After hydrolysis* gram
Adenine picrate	1 115	1 237
Guanine	0 135	0 310
Xanthine	0 0	0 060
Hypoxanthine	0 0	0 0
50 cc for phosphorus		
$Mg\ NH_4\ PO_4\ 6H_2O$	0 592	0 549

The increase in the amount of purine bases by hydrolysis may have been due to the presence of their precursors in the digested product, but it is likely that hydrolysis removed substances affecting the solubility of silver and copper compounds used in the isolation

EXPERIMENT III 100 grams compressed yeast, 300 cc water, 375 mgm guanine chloride digestion, 19 days

250 cc for purine bases	*Without hydrolysis* gram
Adenine picrate	0 111
Guanine	0 0
Xanthine (loss through accident)	0 075
Hypoxanthine	0 0

EXPERIMENT IV 100 grams compressed yeast, 300 cc water digestion, 8 days

	After hydrolysis gram
Adenine picrate	0 365
Guanine	0 064
Xanthine	0 083
Hypoxanthine	0 0

Experiment V 100 grams compressed yeast, 300 cc water, 300 mgm guanine chloride digestion, 8 days

	After hydrolysis gram
Adenine picrate	0 217
Guanine	0 064
Xanthine	0 315
Hypoxanthine	0 0

Experiment VI 150 grams yeast powder, 750 cc water digestion, 10 days

200 cc for purine bases	Without hydrolysis gram	After hydrolysis gram
Adenine picrate	0 141	0 360
Guanine	trace	0 189
Xanthine	0 0	trace
Hypoxanthine	0 0	0 0
20 cc for phosphorus		
$Mg\ NH_4\ PH_46H_2O$	0 347	0 370

Experiment VII 150 grams yeast powder, 750 cc water, 26 5 grams yeast nucleic acid digestion, 10 days

The product proved difficult to handle After a number of attempts at filtration and centrifugation in which a large amount of the material was either lost or abandoned, a small quantity of perfectly transparent fluid was finally obtained, but what proportion of the entire material was contained in this fluid we are unable to state The solution was treated by the method of Levene and Jacobs[6] as follows Neutral lead acetate was added to the boiling hot fluid and the filtrate from the lead precipitate thus formed was treated with additional lead acetate and ammonia This second lead precipitate was decomposed with sulphuretted hydrogen, the filtrate from the lead sulphide evaporated under diminished pressure and the macrocrystalline substance which deposited from the concentrated fluid by cooling to zero was washed first with cold water and then with alcohol After a second crystallization from 80 per cent alcohol the material was dried in a desiccator and weighed The yield was 0 928 gram of guanosine The most perfect crystals of the substance were obtained by treating a hot solution in water or 10 per cent alcohol, with enough acetone to produce a turbidity, and allowing it to cool The substance consisted of perfectly white aggregations of fine needles with pearly lustre and could not be distinguished in any way from guanosine prepared by the neutral hydrolysis of yeast nucleic acid under pressure It did not contain any phosphorus

[6] Levene and Jacobs *Ber d deutsch chem Gesellsch* xlii p 2474 2703, Levene and La Forge *ibid* xliii, p 3150

0.1738 gram substance required 11.33 cc. of standard sulphuric acid (1 cc. = 0.00331 gram nitrogen).

	Calculated for $C_5H_4N_5O.C_5H_9O_4.2H_2O$	Found
N	21.94 per cent	21.52 per cent

500 mgm. of substance were hydrolyzed with 5 per cent sulphuric acid. On cooling, the solution deposited colorless transparent crystals of guanine sulphate, and a drop of the supernatent fluid gave a strong reduction with Fehling's fluid with production of red cuprous oxide (*d*-ribose). The crystals of guanine sulphate were gotten again in solution by heating and the guanine was precipitated with ammonia. 0.238 mgm. guanine was obtained.

Theoretical guanine from guanosine, 47.3 per cent; found, 47.6 per cent.

The guanine after weighing was converted into various derivatives including the characteristic chloride and was thus thoroughly identified.

The ammoniacal filtrate from guanine after removal of the ammonia by boiling produced no precipitate with picric acid. The substance under discussion is guanosine beyond doubt.

We go somewhat into detail concerning this matter because we believe no one has hitherto described the isolation and identification of guanosine formed by the action of ferments on yeast nucleic acid. Of course guanosine could not be formed from thymus nucleic acid. Recently, Schittenhelm, London and Wiener examined the products of the action of intestinal juice on thymus nucleic acid and report the more or less certain finding of guanylic acid, guanosine and adenosine.[7] Levene and Jacobs[8] undertake to clear up the apparent discrepancy in the following language: "Schittenhelm, London and Wiener have thought that they obtained guanosine on the digestion of thymo-nucleic acid by intestinal juice. The substance isolated by them gave the orcin test and had the appearance of guanosine. However, thymonucleic acid contains no pentose and therefore cannot yield guanosine (guanine riboside). Undoubtedly the nucleic acid employed by them was contaminated with guanylic acid. This consideration does not vitiate in any way the conclusion of those writers concerning the mechanism of nucleolysis. . . . ." As we are inclined to believe that the considerations of Levene and Jacobs do rather profoundly affect the conclusions of those writers, we may be permitted to ask if the *adenosine* obtained by Schittenhelm, London and Wiener also undoubtedly resulted from the guanylic acid with which their specimen was contaminated.

[7] *Zeitschr. f. physiol. Chem.*, lxxii, p. 459.

[8] This *Journal*, xii, p. 377.

ANIMAL CALORIMETRY

SEVENTH PAPER

THE METABOLISM OF A DWARF

By F H McCRUDDEN AND GRAHAM LUSK

(From the Physiological Laboratory of the Cornell Medical College, New York City)

(Received for publication, November 13, 1912)

Rubner[1] made experiments upon an American dwarf calling himself "General Mite" who was twenty years old and weighed only 6 6 kilograms This corresponds to the weight of an infant at the breast The individual in question ate a mixed diet and behaved in general like an adult He exhibited himself in public, as a professional freak, dancing and singing He produced daily 531 calories or 80 5 per kilogram of body weight, which may be contrasted with 70 1 calories per kilogram of body weight in an infant at the breast weighing 5 4 kilograms as determined by Rubner and Heubner Per square meter of surface the active dwarf produced 1231 calories whereas the more quiet child produced 1006 An infant given cow's milk produced 1143 calories per square meter of surface Rubner found in these figures the proof that size and not age determined the intensity of the metabolism

EXPERIMENTAL PART

The individual upon whom the following experiments were performed was a patient living at the Rockefeller Hospital for Medical Research who was brought to this laboratory for introduction into the calorimeter His condition was that described by C A Herter[2] as intestinal infantilism His metabolism as indi-

[1] Rubner *Biologische Gesetze*, Marburg, 1887, p 10, *Beitrage zur Ernährung im Knabenalter*, 1902, p 45

[2] Herter *On Infantilism from Chronic Intestinal Infections*, 1908

TAB

Metabolism of

DATE	EXPERIMENT NO	TIME	CO_2	O_2	R Q	H_2O	URINE N	NON PROTEIN CO_2	NON PROTEIN O_2	NON PROTEIN R Q	CALORIES Protein	CALORIES Non protein
1911		*p m*	*grams*	*grams*		*grams*	*gram*	*grams*	*grams*			
April 6	1	1 34–2 34	12 02	11 34	0 77	26 08	0 211	10 05	9 56	0 76	5 59	31 79
		2 34–3 34	11 98	9 66	0 90	26 93	0 211	10 01	7 88	0 92	5 59	27 32
April 7	2	1 34–2 34	12 05	11 27	0 78	17 05	0 230	9 90	9 33	0 77	6 10	31 18
		2 34–3 34	11 52	9 87	0 85	18 74	0 230	9 37	7 93	0 86	6 10	27 06
		a m										
April 8	3	11 10–12 10	10 00	9 52	0 76	16 14	0 162	8 49	8 15	0 76	4 29	27 09
		12 10–1 10	9 90	8 97	0 82	16 88	0 162	8 39	7 60	0 80	4 29	25 45
April 21	4	11 02–12 02	12 67	11 10	0 83	18 91	0 177	11 02	9 60	0 84	4 69	32 60
		12 02– 1 02	11 20	9 36	0 87	24 42	0 177	9 55	7 86	0 88	4 69	26 95
		noon										
May 1	5	12 00–1 00	11 96	10 26	0 85	28 91	0 179	10 29	8 75	0 85	4 74	29 76
		1 00–2 00	11 69	9 55	0 89	24 41	0 179	10 02	8 04	0 91	4 74	27 79

cated by analyses of his urine and feces has already been elsewhere reported by one of the writers[3] The individual J P was seventeen years old, 113 3 centimeters high and weighed 21 3 kilograms naked This gave him a calculated surface area of 0 946 square meters He was accustomed to laboratory procedures Introduction of the electrical resistance rectal thermometer and the subsequent hours spent in the calorimeter were in no way disturbing to him He lay on a mattress placed on the floor of the calorimeter, his head resting on a pillow He was provided with illustrated periodicals which he usually read during the first hour of the

[3] McCrudden *Journ of Exp Med*, xv, p 107, 1911, McCrudden and Fales *ibid*, xv, p 113, 1912

LE I

a dwarf J P

Calories: Total Calculated	Calories: Total Found	Body temperature: Start	Body temperature: End	Body temperature: Difference	Morning weight	Behavior	Food
					kgm		
37 38	36 68	37 40	37 31	−0 09	21 3	Asleep 12 min	No breakfast At 11 30 a m 20 grams rice made into a pudding with cream and sugar
32 91	32 72		37 10	−0 21		Asleep or quiet	
70 29	69 40						
37 28		37 19	37 03	−0 16		Quiet and reading	No breakfast At 11 30 a m 123 grams tenderloin steak (= 4 2 grams N) Refused to eat more
33 16			36 92	−0 11		Quiet	
70 44							
31 38		37 28	37 15	−0 13		Quiet	No breakfast No food
29 74			36 95	−0 20		Quiet	
61 12							
37 29	32 33	37 26	37 20	−0 06		Quiet and reading	Breakfast at 6 30 a m One glass milk toast and one half an orange
31 64	34 70		37 08	−0 12		Quiet	
68 93	67 03						
34 50	33 98	36 91	36 47	−0 44		Awake	Customary breakfast (no record)
32 53	30 87		36 31	−0 16		Awake and asleep	
67 03	64 85						

experiment after which he fell asleep or remained perfectly quiet

The analytical results were obtained at an environmental temperature of 26° to 27° and are presented in Table I

The basal metabolism

As in the case of the determination of the basal metabolism of Dog II, this experiment was made about eighteen hours after the ingestion of the last meal The dwarf partook of no breakfast He complained a little of weakness He was quiet throughout the experiment, the results of which were as follows

Experiment 8 Metabolism of J P, eighteen hours after food ingestion

TIME	URINE N	NON-PROTEIN R Q	CALORIES Protein	Non protein	Total calculated
a m					
11 10–12 10	0 162	0 76	4 29	27 09	31 38
12 10– 1 10	0 162	0 80	4 29	25 45	29 74
					61 12

These figures show an average basal metabolism of 30 56 calories per hour Calculated for the day the total heat production would be 733 calories, or 29 per kilogram of body weight and 775 per square meter of body surface This last figure corresponds to those representing the basal metabolisms of Dog I (weight = 13 8 kgm) and Dog II (weight = 9 3 kgm) which were 759 and 784 respectively All these figures agree within 3 per cent and the results show the verity of Rubner's law of skin area but place the amount of heat eliminated at a lower level than Rubner's figures indicate This is because the experiments were performed when food was absent from the intestine and a condition of complete rest and absence from thermal influences prevailed Similar results may be calculated from Rubner's[4] own observations (Experiments 39–40) on a well-trained quiet dog weighing 4 6 kilograms which, during the first and second days of fasting, was kept at an environmental temperature of 33° The surface area of this dog may be calculated to be 0 3079 square meter and the heat production at 746 and 752 calories per square meter of surface on the first and second days of fasting

It is apparent from this that the *basal metabolism* is a truer starting point for comparative studies as regards the law of skin area than are experiments which include mechanical work, thermal influences and the results of food ingestion

From Benedict's monograph on Inanition,[5] one may estimate the basal metabolism in man as calculated from a night period extending from 1 00 to 7 00 a m and beginning thirty hours after the last ingestion of food The following table shows the basal

[4] Rubner *Die Gesetze des Energieverbrauchs*, 1902, p 323

[5] Benedict *Metabolism in Inanition*, Carnegie Institution of Washington, 1907, pp 480–83

metabolism of various individuals, founded on the heat production per square meter of surface calculated for twenty-four hours

Table showing calories per square meter of surface in twenty-four hours based upon the minimal night metabolism from the thirtieth to thirty-sixth hours of fasting in man

EXPERIMENT NUMBER	INDIVIDUAL	BODY WEIGHT	PER SQUARE METER OF BODY SURFACE	BEHAVIOR
		kgm	*calories*	
59	B F D	67 8	834 8	Good sleep till 6 35 a m
68	A L L	72 9	809 5	Asleep
69	A L L	73 8	905 7	Not stated
71	S A B	58 2	771 8	Asleep
73	S A B	59 1	856 3	Asleep till 3 30 then awake every hour
75	S A B	59 5	858 2	Slept fairly well
77	S A B	61 6	893 8	Felt too warm all night
79	H E S	57 2	1015	Got up at 2 24 a m thinking it morning Sick at stomach when he got up
80	C R Y	69 3	940 4	No record
81	A H M	62 0	738 8	Slept well all night
82	H C K	71 5	886 2	Awoke early
83	H R D	55 6	954 4	Sound sleep Awake once
85	N M P	67 6	935 1	Woke up twice Pneumograph uncomfortable
89	D W	79 1	865 6	Woke up at 3 45 a m Dozed after this

Benedict on p 56 states of the experiment on A L L "fasting experiment made under conditions ideal for obtaining minimum muscular activity" and on p 107 he describes subject S A B as being of "highly nervous temperament"

In experiments 59, 68, 71, 81 and 83 the individuals slept soundly all night In the last named experiment (No 83), the heat production was exceptionally high, 954 calories per square meter of surface In the other four experiments the values 835, 809, 772 and 739 are found to average 789 calories per square meter of surface The variation amounts to the considerable figure of 13 per cent, but the average is equal to the heat production found in our experiments

The total heat production of the dwarf, therefore, conforms to Rubner's general law of skin area, which is a fundamental law

of metabolism Only in the case of infants has exception to this law been found Howland[6] has shown that the metabolism of sleeping infants eighteen hours after food ingestion reaches 1100 calories per square meter of surface, indicating a higher heat production in the youthful protoplasm than is present in the adult

As regards the kind of energy yielding material oxidized by the dwarf, it is noted that 14 per cent of the energy of metabolism is derived from protein and 86 per cent from fat and carbohydrate, a proportion which is entirely normal

The effect of food ingestion

Several experiments were performed to note the effect of food ingestion On one morning no breakfast was taken until 11 30 a m when a large tenderloin steak was given Of this only the equivalent of 123 grams of fresh lean meat was eaten, with a nitrogen content of 4 2 grams This small quantity hardly raised the nitrogen elimination in the urine and caused no pronounced effect upon metabolism A summary of all these experiments is presented below

Metabolism of J P after food ingestion

EXP NO	TIME	URINE N	NON-PROTEIN R Q	CALORIES Protein	Non-protein	Total calculated	Total found	FOOD
	p m							
1	1 34–2 34	0 211	0 76	5 59	31 79	37 38	36 68	Carbohydrate and fat at 11 30 a m
	2 34–3 34	0 211	0 92	5 59	27 32	32 91	32 72	
						70 29	69 40	
2	1 34–2 34	0 230	0 77	6 10	31 18	37 28		Meat at 11 30 a m
	2 34–3 34	0 230	0 86	6 10	27 06	33 16		
						70 44		
	a m							
4	11 02–12 02	0 177	0 84	4 69	32 60	37 29	32 33	Breakfast at 6 30 a m
	12 02– 1 02	0 177	0 88	4 69	26 95	31 64	34 70	
						68 93	67 03	
	noon							
5	12 00–1 00	0 179	0 85	4 74	29 76	34 50	33 98	Breakfast
	1 00–2 00	0 179	0 91	4 74	27 79	32 53	30 87	
						67 03	64 85	

[6] Howland *Zeitschr f physiol Chem*, lxxiv, p 1, 1911

In the four experiments cited above the calories of the protein metabolism amount to 16, 17, 14 and 15 per cent of the total metabolism which indicates the presence of entirely normal conditions

The results presented naturally fall into two divisions, (1) the first hours during which illustrated papers or a book were being read prior to complete relaxation or sleep and (2) the second hours during which the boy was quiet or asleep throughout the period

During three experiments the calculated heat production during the first hour was 37 38, 37 28 and 37 29 calories per hour, an average of 37 33 calories During the second more quiet hours the heat production fell to 32 91, 33 16, 31 64 and 32 53 calories, an average of 32 55 Comparing these figures with the value of the basal metabolism the following results are obtained

	CALORIES	INCREASE	
		Calories	Per cent
Basal metabolism	30 56		
Metabolism resting in bed after food	32 55	+1 99	6 6
Metabolism reading papers in bed	37 33	+4 78	14 7

The experiments therefore led to the conclusion that the influence of small quantities of ingested food is to increase the metabolism by 6 6 per cent, whereas muscular work attendant upon reading in recumbent position causes a still further increase of 14 7 per cent The influence of very moderate muscular activity is shown pronouncedly

Calculated on the basis of twenty-four hours the metabolism may be measured as follows

	CALORIES IN 24 HOURS		
	Total	Per kilogram	Per square meter of surface
Basal metabolism	733	33	775
Metabolism, reading after food	896	42	947

The authors gratefully acknowledge the assistance of Dr H B Williams and Mr J A Riche in the performance of this work

CONCLUSIONS

A dwarf, suffering from infantilism, seventeen years old and weighing 21 3 kilograms had a basal metabolism of 775 calories per square meter of surface in twenty-four hours which may be contrasted with 759 and 784 in two dogs of different weights

The basal metabolism was increased 6 6 per cent as a result of the ingestion of food, and this again was increased by 14 7 per cent when the boy was reading illustrated periodicals in bed

The protein metabolism yielded the normal proportion of 15 per cent of the total calories of heat production

Nothing abnormal could be detected in the metabolic processes of the individual, as determined from these calorimetric observations

ON THE REFRACTIVE INDICES OF SOLUTIONS OF CERTAIN PROTEINS

VIII GLOBIN

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(Received for publication, November 16, 1912)

A THE PREPARATION OF GLOBIN [1]

Globin was prepared from ox corpuscles by three different modifications of the method of Schulz[2] as follows

A thick suspension of ox corpuscles was obtained from freshly defibrinated ox blood by centrifugalization, the volume of the suspension being about one-third that of the blood from which it was derived

After pipetting off the supernatant serum the suspension was diluted to the original volume of the blood from which it was derived by the addition of $\frac{M}{6}$ NaCl solution, and the centrifugalization was repeated, the supernatant fluid being removed as before This was repeated six times in order to free the corpuscles from adherent serum After the last centrifugalization the corpuscle suspension was not diluted again with NaCl

The thick suspension of corpuscles which was thus obtained was diluted to ten times its volume by the addition of distilled water, the corpuscles were thus "laked" and the contained haemoglobin was discharged into the water, forming a clear solution which was allowed to stand in tall glass vessels for twenty-four hours in order to permit the leucocytes to settle The upper portion of the fluid was then decanted and employed, the lower portion of the fluid being rejected

[1] I am indebted to Dr Charles B Bennett for assistance in some initial attempts to prepare this substance

[2] Fr N Schulz *Zeitschr f physiol Chem* , xxiv, p 449, 1898

It has been shown by Preyer,[3] Schulz[4] and others that the addition of a sufficient concentration of acid to a solution of haemoglobin results in its decomposition into haematin and a protein, globin The former of those substances is readily soluble in acidified ether, hence this decomposition affords a means of preparing globin and separating it from haematin Schulz points out, however, that the concentration of acid employed to bring about the decomposition of the haemoglobin is of great importance in determining the completeness or otherwise of the subsequent extraction of the haematin by ether, and my own experiments abundantly confirm this observation If too small a quantity of acid be employed the haematin is only very incompletely removed from the aqueous layer by extraction with ether If too large a quantity of acid be employed the globin is denatured and forms with the ether a species of gel leaving the subnatant aqueous layer clear and colorless but free from globin[5] I have in no instance been able to obtain a perfectly colorless aqueous layer which still contains globin in a form precipitable by ammonia

Preliminary experiments with the solution of corpuscles described above showed that the addition of 0 25 equivalent of HCl per liter afforded the best results The aqueous layer was still considerably colored, but 0 38 equivalent, while permitting almost all the color to be extracted from the aqueous layer, also caused the greater part of the globin to leave the aqueous layer[6] On the other hand, 0 13 equivalent left the aqueous layer, after extraction with ether, very deeply colored and the separation into two layers was also very incomplete

Accordingly 2 5 liter portions of the corpuscle solution were placed in six-liter bottles, to the contents of each bottle were added 56 cc of concentrated HCl (specific gravity 1 18) and the mixture was thoroughly shaken and allowed to stand at room

[3] Preyer *Die Blutkrystalle*, Jena, 1871

[4] Fr N Schulz *loc cit*

[5] That is, failing to yield a precipitate upon the addition of ammonia

[6] Dr Charles B Bennett finds that once the decomposition of the haemoglobin into globin and haematin hydrochloride has been brought about by hydrochloric acid, calcium chloride added to the aqueous layer has the same effect upon the partition of haematin and globin between the ether and water as hydrochloric acid itself

temperature for one hour The addition of the acid caused a flocculent precipitate to appear and the mixture turned dark brown Two and a half liters of ether were then added to each bottle and the contents were shaken thoroughly until they attained a thick oily consistency Rubber stoppers with two perforations were then fitted into the necks of the bottles Through one perforation was inserted a long glass tube reaching to the bottom of the bottle, to act subsequently as an air inlet, and through the other was inserted a short tube, just reaching to the bottom of the stopper and provided with a rubber tube and pinchcock The stoppers were then tied down and the bottles quickly inverted and allowed to stand at room temperature for twenty-four hours

By the following day the contents of the bottles had separated into two layers, an upper, jelly-like, very deeply colored ether layer and a lower, still somewhat deeply colored aqueous layer measuring about 1 7 liters The latter was drawn off through the shorter of the two tubes inserted through the stoppers

An attempt was made to further decolorize this fluid by adding to it more acid and then repeating the extraction with ether, with the very peculiar result that the addition of more acid, far from favoring the extraction of haematin by the ether, was found to actually *diminish* the amount of haematin taken up by the ether In fact the addition of 0 05 or 0 10 equivalent per liter of HCl and subsequent extraction with an equal volume of ether resulted in a very rapid separation into an almost colorless ether layer and a still highly colored water layer, yet, as the experiments cited *above show, had this acid been added before the first extraction* it would have led to a more complete extraction of haematin and, in addition, to the inclusion in the jelly-like ethereal layer of the greater part of the globin It seems that the ethereal extract of the original solution of blood-corpuscles must contain some substance or substances which enhance the solubility of haematin hydrochloride in ether and that when these are removed, although de-emulsification of the ether solution mixture is accelerated by acid, the power of the ether to take up haematin from the water is diminished

On shaking up the still dark-colored solution with ether *without* any further addition of acid, de-emulsification of the mixture was extremely slow I accordingly adopted the device employed

by Schulz,[7] namely, that of adding to the ether one-quarter of its volume of alcohol This resulted in quick separation of the mixture, after shaking, into two layers, an upper, ether-alcohol layer which was deeply colored and a lower aqueous layer somewhat less colored than before

To 2 liters of the watery layer derived from the first extraction were added 2 liters of ether and 500 cc of alcohol, and the mixture was thoroughly shaken After allowing it to stand for a short period the watery layer was drawn off and the extraction repeated twice After the last extraction the watery layer had become a pale opaque brown and the ether layer was only slightly colored The watery layer was now diluted to ten times its volume by the addition of distilled water, and 150 cc of a 20 per cent solution of ammonia were added A precipitate appeared which quickly redissolved but on adding a nearly equivalent amount of HCl it reappeared, thus confirming the finding of Schulz that globin is insoluble in dilute ammonia in the presence of a sufficiency of ammonium chloride This precipitate was collected upon a hardened filter-paper, washed in large volumes of alcohol and ether[8] and dried over H_2SO_4 at 36° After twenty-four hours it was pulverized, passed through a fine sieve and returned to the incubator to dry over H_2SO_4 for one week The yield from 2 liters of the aqueous layer obtained after the first extraction, corresponding to 3 liters of the original corpuscle solution or 300 cc of the corpuscle suspension (= about 900 cc of blood) was 4 2 grams

Globin was thus obtained in the form of a light very friable greyish powder, readily soluble in dilute ($\frac{N}{10}$) acids and alkalies,

[7] *Loc cit*

[8] According to Schulz (*loc cit*), globin, after washing with alcohol and ether and drying, is much less soluble in dilute alkalies and acids than normal globin I have not observed this It is true that dry globin dissolves much more slowly than wet globin, but this is a perfectly general phenomenon with proteins (cf T Brailsford Robertson *Die physikalische Chemie der Proteine*, Dresden, 1912, p 75) and is due simply to the fact that time is consumed in the penetration of the dry sponge-like particles of dehydrated protein by the solvent (Cf T Brailsford Robertson *ibid*, p 249) I find, however, that if dry globin be brought into contact with dry *acidified* alcohol containing 0 1 equivalent of HCl per liter (prepared by passing dry HCl gas into absolute alcohol) not only does it fail to dissolve but it is rendered insoluble in dilute aqueous solutions of acids

yielding, at a concentration of 2 per cent, light brown solutions This substance will be referred to as Preparation I

I observed that on adding to the watery layer obtained from the first extraction four volumes of a mixture of equal parts by volume of alcohol and ether, a light-colored precipitate of globin resulted, leaving the fluid very deeply colored, although the addition of four volumes of alcohol alone did not produce a precipitate Accordingly to 1-liter portions of the fluid derived from the first extraction I added 2 liters of ether and 2 liters of alcohol After allowing the precipitate to settle, which it did with tolerable rapidity, and decanting the greater part of the supernatant fluid, it was collected on a hardened filter, thoroughly drained, washed twice with 1 liter of alcohol and twice with 1 liter of ether within an incubator at 36° over H_2SO_4 and then dried in the incubator for from eighteen to twenty-four hours, at the end of which time it was pulverized, sifted and then returned to the incubator to dry for a week

Three 1-liter portions of the watery layer derived from the first extraction with ether were treated in this manner The total yield from this fluid, corresponding to 4500 cc of the original corpuscle solution, 450 cc of the corpuscle suspension and about 1350 cc of blood, was 37 grams

Globin was thus obtained in the form of a coarse brown powder, readily soluble in dilute acids and alkalies, yielding, at a concentration of 2 per cent, solutions somewhat more highly colored than those yielded by the preparation previously described This substance will be referred to as Preparation II

The alcohol-ether precipitate of globin, prepared in the manner described above, is readily soluble in dilute HCl and precipitated from this solution by ammonia

Accordingly the globin from 1-liter portions of the watery layer derived from the first extraction with ether was precipitated by four volumes of alcohol-ether mixture, the precipitate collected upon a hardened filter, washed with 1 liter of alcohol, drained and then scraped off the paper and dissolved in 1 liter of $\frac{N}{10}$ HCl This solution was then diluted to 4 liters, and 20 per cent ammonia solution carefully added until a precipitate just appeared (11 cc) One cubic centimeter more of the strong ammonia was then added and the dense flocculent precipitate collected upon a hardened

filter it filtered rapidly, the filtrate being pale yellow. The precipitate was then washed in alcohol (1 liter), alcohol-ether mixture (1 liter) and ether (2 liters), the washings with alcohol-ether mixture and ether being conducted within an incubator at 36° over H_2SO_4. After draining, the precipitate was allowed to dry for twenty-four hours and then pulverized and sifted and returned to the incubator to dry over H_2SO_4 for one week.

Two 1-liter portions of the water layer derived from the first extraction with ether were treated in this manner. The total yield from this fluid, corresponding to 3 liters of the original corpuscle solution, 300 cc. of the corpuscle suspension and about 900 cc. of blood, was 20.6 grams.

Globin was thus obtained in the form of a very pale saffron-colored, light, friable powder, readily soluble in dilute acids and alkalies, yielding at a concentration of 2 per cent, light brown solutions *less colored than those yielded by either of the preparations* previously described. This substance will be referred to as Preparation III.

Schulz thus describes the product which he obtained, starting with crystallized haemoglobin: "Ein gelbliches, sehr lockeres, nicht wesentlich hygroskopisches Pulver." It differs from the majority of proteins by its high content of carbon (= 54.97 per cent, Schulz). It is predominantly basic and is considered by Schulz to be a histone. According to Abderhalden[9] the globin from horse blood yields, on cleavage, notable quantities of leucine (29 per cent) and histidine (10.96 per cent.)

B. THE DETERMINATIONS OF REFRACTIVITY

Two per cent solutions were made up of each of the above preparations in $\frac{N}{10}$ KOH, and portions of these solutions were diluted to 1 per cent by the addition of $\frac{N}{10}$ KOH. Two and one per cent solutions of Preparation III in $\frac{N}{10}$ HCl were also prepared. The refractive indices of these solutions and of the solvents ($\frac{N}{10}$ KOH and $\frac{N}{10}$ HCl) were measured at 18°C, in a Pulfrich refractometer, using *d*-sodium flame as the source of light.

[9] E. Fischer and E. Abderhalden: *Zeitschr. f. physiol. Chem.*, xxxvi, p. 268, 1902; E. Abderhalden: *ibid.*, xxxvii, p. 484, 1903; E. Abderhalden and L. Baumann: *ibid.*, li, p. 397, 1907.

The following were the results obtained The values headed a are calculated from the formula $n - n_1 = a \times c$ where n is the refractive index of the solution, n_1 that of the solvent and c is the percentage of protein in the solution

TABLE I

SOLUTION	n = REFRACTIVE INDEX OF SOLUTION AT 18 C	a
$\frac{N}{10}$ KOH	1 33465	
$\frac{N}{10}$ HCl	1 33442	
1 per cent Preparation I in $\frac{N}{10}$ KOH	1 33623	0 00158 ± 0 00008
2 per cent Preparation I in $\frac{N}{10}$ KOH	1 33791	0 00163 ± 0 00004
1 per cent Preparation II in $\frac{N}{10}$ KOH	1 33607	0 00142 ± 0 00008
2 per cent Preparation II in $\frac{N}{10}$ KOH	1 33751	0 00143 ± 0 00004
1 per cent Preparation III in $\frac{N}{10}$ KOH	1 33631	0 00166 ± 0 00008
2 per cent Preparation III in $\frac{N}{10}$ KOH	1 33799	0 00167 ± 0 00004
1 per cent Preparation III in $\frac{N}{10}$ HCl	1 33615	0 00173 ± 0 00008
2 per cent Preparation III in $\frac{N}{10}$ HCl	1 33783	0 00171 ± 0 00004

It will be observed that in each case, within the experimental error (due to a possible error of ± 1′ in reading the angle of total reflection), the difference between the refractivity of the solutions and that of the solvent is directly proportional to the concentration of protein dissolved in the solution The values of a for the various preparations, however, differ somewhat In order to assign to each observation its due weight in determining the average values of a for each of these preparations it is necessary to add together the observed values of $n - n_1$ for the different solutions (2 per cent and 1 per cent) of each preparation and divide this sum by the sum of the concentrations employed (= 3) Proceeding in this way we obtain

TABLE II

SUBSTANCE	VALUE OF a
Preparation I dissolved in $\frac{N}{10}$ KOH	0 00161 ± 0 00005
Preparation II dissolved in $\frac{N}{10}$ KOH	0 00142 ± 0 00005
Preparation III dissolved in $\frac{N}{10}$ KOH	0 00167 ± 0 00005
Preparation III dissolved in $\frac{N}{10}$ HCl	0 00171 ± 0 00005

The values of a for Preparation III dissolved in $\frac{N}{10}$ KOH and in $\frac{N}{10}$ HCl are identical within the experimental error, their aver-

age is 0.00169 ± 0.00005. The value of a for Preparation I is slightly lower, while that of a for Preparation II is very much lower. These values obviously correspond with the relative purities of the preparations. It may be assumed, since both of them are of a very light color, that Preparations I and III are both very nearly pure, and, as we see, their refractivities are nearly equal. Preparation III, however, having been precipitated twice by different methods is less likely to be contaminated by impurities than Preparation I. On the other hand it is evident from its dark color that Preparation II is impure, and we see that its refractivity departs widely from that of Preparation III or that of Preparation I.

It appears probable, therefore, that the average value of the refractivity per gram per 100 cc. of solution of Preparation III determined above is, within the experimental error of the estimation, the refractivity, per gram per 100 cc. of solvent, of pure globin.

SUMMARY

Globin has been prepared from ox corpuscles by three different modifications of the method originally employed by Schulz.

The value of a (= change in the refractive index of the solvent due to the introduction of 1 per cent of the protein) for the purest preparation obtained, when dissolved in $\frac{N}{10}$ KOH or $\frac{N}{10}$ HCl, is 0.00169 ± 0.00005.

Less pure preparations yielded lower values of a.

THE SULPHATIDE OF THE BRAIN

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(Received for publication, November 21, 1912)

There exists very little definite information regarding the nature of the sulphur-containing lipoids The authors who are defending the individuality of protagon assume that both sulphuric and phosphoric acids enter into the structure of its molecule On the other hand, Thudichum,[1] on the ground of theoretical considerations, was led to the belief that the sulphatide is a distinct lipoid having some properties in common with the phosphatides Unfortunately he failed to separate the two substances The ratio of sulphur to phosphorus in the purest sample analyzed by this author was 3 2, in other samples the ratio was 1 1

In very recent years W Koch[2] made the sulphatide the subject of a new investigation Koch arrived at the conclusion that the sulphatide contained an equimolecular proportion of phosphoric and of sulphuric acids The analytical data led him to formulate the structure of the substance in the following manner

$$(C_{41}H_{29}NO_8) - O - (SO\ ) - O - (C_{62}H_{104}N_2PO_4)$$

or, more graphically,

$$\begin{array}{c} \mathrm{O} \\ \| \\ \text{Phosphatide} - \mathrm{O} - \mathrm{S} - \mathrm{O} - \text{Cerebroside} \\ \| \\ \mathrm{O} \end{array}$$

Thus the "phosphatide-cerebroside-sulphatide" of Koch contained all the elements that were supposed to be parts of protagon, and in a way the substance may have been regarded with some degree of justice as the purest protagon, although W Koch militated against the existence of this complex

[1] *A Treatise on the Chemical Constitution of the Brain*, London, 1884

[2] *Zeitschr f physiol Chem* , lxx p 94, 1910

In course of an investigation into the lipoids of the brain we found it possible to isolate the sulphur-containing lipoid of the brain entirely free from phosphatides In its physical appearance and in many of its properties the substance possessed a great resemblance to other lipoids and had the following composition

0 1216 gram of the substance gave 0 2714 gram CO_2 and 0 1160 gram H_2O
0 1216 gram of the substance gave 0 2706 gram CO_2 and 0 1150 gram H_2O
0 2000 gram of the substance employed for Kjeldahl nitrogen estimation required for neutralization 3 3 cc of $\frac{N}{10}$ sulphuric acid
0 4000 gram of the substance used for a sulphur estimation gave 0 0766 gram of barium sulphate
0 4000 gram of the substance used for phosphorus estimation gave a negative result

A comparison of the results of the analysis of the samples here described, and of those obtained by Thudichum and by Koch shows the following results

	THUDICHUM	KOCH	LEVENE
C	47 70		60 90
H	7 54		10 67
N	1 14		2 31
P	3 94	1 80	0 00
S	6 19	1 91	2 66
O	33 49		23 46

The substance is dextrorotatory and melts at 210° C (uncor)

By the method employed for the preparation and purification of the sulphatide it was not possible to further purify the substance Other methods will have to be devised for that purpose

It must be remarked here that both Thudichum and Koch found that human brain offered a more suitable material for the preparation of their sulphatide The substance described in this communication was isolated from beef brains

Further work on the chemical structure of the substance is in progress

A NOTE ON THE SUGAR TOLERANCE IN THE PIG

By A J CARLSON and F M DRENNAN

(*From the Hull Physiological Laboratory of the University of Chicago*)

(Received for publication, November 27, 1912)

According to Minkowski[1] almost complete pancreatectomy in the pig does not result in as severe diabetes as is the case in other mammals The results obtained by Gould and Carlson[2] with almost complete pancreatectomy in two pigs seemed to confirm Minkowski's observation In order to determine whether the pig's tissues possess unusual power to utilize sugars we made the following comparisons of the tolerance of two pigs before and after complete pancreatectomy Three pigs of nearly the same size and age were selected One of these was kept as control on the normal rate of growth

Pig No I

Weight, 5 kgm

1911, October 15–20 Dextrose dissolved in milk in quantities varying from 2 5 to 10 grams was given on empty stomach *In every case sugar appeared in the urine within one hour after giving the sugar by mouth* The total quantity of sugar excreted was not determined The urine did not contain sugar when the pig was fed on milk unless dextrose was given by mouth

October 22 Extirpation of the pancreas completed at 4 p m Urine was voided at 6, at 8 and at 10 p m All the samples contained sugar

October 23 170 cc of urine passed in twenty-four hours, containing 10 20 grams of sugar

October 24 *7 a m* 80 cc of urine, containing 5 grams of sugar Animal died of peritonitis at 11 a m Post mortem showed complete removal of the pancreas

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[1] Minkowski *Arch f exp Path u Pharm* , xxxi, p 93, 1893

[2] Gould and Carlson *Amer Journ of Physiol* , xxix, p 174, 1911

PIG No II

October 25–28 Milk diet Quantity of urine during twenty-four hours on milk diet, 350–400 cc

October 29 *10 30 a m* Fresh sample of urine contained no sugar

11 00 a m 5 grams of dextrose in 50 cc of buttermilk given by mouth

12 30 p m 50 cc of urine (2 urinations) contained 0 531 gram of sugar

1 45 p m 45 cc of urine, no sugar

2 30 p m 35 cc of urine, no sugar

October 30 *12 m* Fresh sample of urine contained no sugar 5 grams of dextrose in 50 cc of buttermilk given by mouth

1 00 p m 40 cc of urine contained 0 216 gram of sugar

2 00 p m 15 cc of urine contained 0 203 gram of sugar

2 30 p m 20 cc of urine contained 0 188 gram of sugar

Total sugar = 0 607 gram

October 31 *9 30 a m* Sample of urine free from sugar

10 00 a m 2 5 grams of dextrose in 50 cc of buttermilk given by mouth

10 20 a m Sample of urine contained no sugar

11 00 a m 30 cc of urine contained 0 133 gram of sugar

12 00 m 28 cc of urine contained 0 250 gram of sugar

12 30 p m Sample of urine free from sugar

Total sugar = 0 383 gram

During the time October 29–31 the pig was given no food except the quantities of dextrose and buttermilk shown above

October 31 *1 00 p m* The pig was allowed to eat as much as it desired of a mixture of bread and sweet milk

2 00 p m 40 cc of urine contained 0 68 gram of sugar

November 5 *10 00 a m* The pig was allowed to eat as much as it desired of cooked corn meal

11 00 a m 50 cc of urine contained 0 312 gram of sugar

11 30 a m 50 cc of urine contained 0 412 gram of sugar

12 00 m 35 cc of urine contained 0 143 gram of sugar

1 00 p m 100 cc of urine contained slight traces of sugar

Total sugar = 0 87+ gram

November 12 After starving for two days the pig was allowed to eat as much as it desired of cracked corn No sugar appeared in the urine

November 21 Weight of pig, 8 2 kgm Extirpation of the pancreas During November 22–28 the daily ration consisted of 75 grams of bread in buttermilk

November 22 310 cc of urine contained 23 grams of sugar

November 23 350 cc of urine contained 58 grams of sugar

November 24 605 cc of urine contained 92 grams of sugar

November 25 460 cc of urine contained 59 grams of sugar

November 26 835 cc of urine contained 114 grams of sugar

November 27 900 cc of urine contained 128 grams of sugar

November 28 920 cc of urine contained 126 grams of sugar

From November 29 to January 8 the food consisted of bread and milk The sugar excretion in the urine continued about the same as during the

week of November 22–28, but the pig was put in the metabolism cage each day only for the time required to secure one sample of urine

January 8 The pig weighed 6 2 kgm , having lost 2 kgm since November 21 When the pig was placed in the cage 450 cc of urine were voided, in two hours, containing 52 grams of sugar The pig was given nothing but buttermilk for forty-eight hours

January 10 270 cc of urine voided in three hours, containing 30 grams of sugar The bread and buttermilk ration was resumed

January 12 937 cc of urine contained 113 grams of sugar The feces contained no starch

January 18 1000 cc of urine Total sugar = 110 grams, total N = 11 58 grams

January 21 After feeding 200 grams of bread in 500 cc of buttermilk, 1300 cc of urine Total sugar = 151 grams, Total N = 14 1 grams

No food was given January 21–24

January 22 830 cc of urine Total sugar = 71 grams, total N = 7 72 grams

January 23 1410 cc of urine Total sugar = 67 7 grams, total N = 12 0 grams Weight of pig, 5 72 kgm

The pig did not seem to recover well after the starvation period, January 21–24 The animal did not eat heartily and was lying down most of the time On January 26, the animal refused food and water, so it was killed with ether Weight, 5 60 kgm

Post mortem findings The pancreas was completely removed The stomach was unusually dilated and contained 2280 cc of fluid and ingested material There was a large aneurism sac in the fundus (greater curvature) where the muscle layers were absent The sac had been formed by a rupture of the muscle layers The duodenum was greatly dilated and filled with fluid The common bile duct was dilated to a diameter of 1 cm

Pig No III Control

Several tests were made of the tolerance to dextrose *per os* with results identical to those reported in the cases of pig I and II On November 21 (date of pancreatectomy of pig II, weighing 8 2 kgm) pig III weighed 8 kgm On January 27 this pig weighed 27 kgm From November 21 to January 26 the diabetic pig lost weight at the rate of about 25 grams per day, while the normal pig gained at the rate of 300 grams per day The actual loss of body materials by the diabetic pig was greater than these figures indicate, for more than a third of the weight of the pig at death represented fluid and ingested material in the enormously dilated stomach and duodenum

1 While the results on pig II show that complete pancreatectomy is followed by the fatal diabetes typical of other species, we were impressed by the length of time the pig continued in fair condition and vigor It is probable that the pig would have

lasted ten to twenty days longer if it had not been weakened by the three days' fast, January 21–23

We have no explanation for the dilated condition of the stomach and the duodenum, unless it be essentially a mechanical effect, that is, an insatiate appetite resulting in ingestion of too great quantities of food for the weakened stomach walls

2 The normal pig has a lower tolerance for dextrose, bread or cooked starch given by mouth than any species so far studied When a normal pig weighing 8–10 kilos shows marked alimentary glycosuria when given 2 5 grams of dextrose, and that on an empty stomach, the lack of tolerance seems to border on the pathological The lack of tolerance is, of course, due to failure of adjustment of the rate of absorption to the rate of fixation or storage of the sugar We had planned to test the parenteral sugar tolerance in the pig, but that seemed useless on finding the slight tolerance *per os*

This condition in the pig must lead to waste of calories in proportion to the quantity of bread or cooked starch fed

3 It might be pointed out that the pig is a very convenient subject for physiological work involving frequent collections of the urine The pig urinates on the average every thirty or forty-five minutes The animal is almost as accommodating as if one had direct access to the ureters

A NEW (COLORIMETRIC) METHOD FOR THE DETERMINATION OF URIC ACID IN BLOOD

By OTTO FOLIN AND W DENIS

(*From the Biochemical Laboratory of Harvard Medical School, Boston*)

(Received for publication, November 30, 1912)

Notwithstanding the great physiological and clinical interest which for more than a hundred years has been attached to the "uric acid in blood" no suitable method for the determination of this uric acid has yet been discovered The ammoniacal silver nitrate method of Salkowski and the cupric bisulphite method of Kruger for precipitating uric acid have indeed rendered valuable service, for by their means the presence of traces of uric acid in certain kinds of pathological blood has been positively established Rough quantitative determinations of the uric acid in such blood have also been made on the basis of those methods though it is very doubtful how trustworthy have been the results

Brugsch and Schittenhelm clearly recognize the doubtful character of their own quantitative uric acid determinations in blood on the basis of those methods,[1] and in fact regard the determination of as little as 1-3 mgm of uric acid as practically impossible They emphasize the value of the qualitative test for uric acid by the murexide test, a point which is well taken in view of the fact that the analytical methods are not sensitive enough to yield positive qualitative tests for the uric acid of normal blood

An astounding feature of the procedure described by Brugsch and Schittenhelm for the detection and quantitative estimation of uric acid in blood is the recommendation[2] that the blood be treated with hot caustic potash solution (2-5 per cent) in connection with the preliminary precipitation of the proteins Such a treatment with caustic alkalies is absolutely fatal since it would destroy anywhere from 30 per cent to all of the uric acid originally present

In view of the well-known ease with which uric acid decomposes in alkaline solutions such a recommendation coming from Schittenhelm, who for

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[1] *Zeitschr f exp Path u Ther* , iv, p 440, 1907, *Münch med Wochenschr* lix, p 2377, 1912

[2] *Zeitschr f exp Path u Ther* , iv, p 441, 1907

years has been an active contributor in the field of the autolytic formation and destruction of uric acid and other purines, is certainly surprising

As far back as 1901 Folin and Shaffer called attention to the great instability of uric acid in alkaline solutions and cited figures to show that over 30 per cent is lost when uric acid is heated on the water bath for forty five minutes with 15 per cent sodium hydrate solutions[3] Folin and Shaffer traced the decomposition of the uric acid by titrations with potassium permanganate We have now made another series of tests by the new colorimetric determination described in the last number of this *Journal* and from our results cite the following

1 A 2 per cent NaOH solution containing 10 mgm uric acid per 20 cc lost 35 per cent by boiling three minutes

2 A corresponding (2 per cent) Na_2CO_3 solution under the same conditions lost 15 per cent of the uric acid

3 A 0 5 per cent NaOH solution in three minutes produced a loss of 18 per cent of the uric acid

4 A 0 5 per cent Na_2CO_3 solution in three minutes destroyed 12 per cent of the uric acid

We venture to suggest that the reason why Schittenhelm has overlooked the instability of uric acid is the fact reported by E Fischer that uric acid solutions do not lose ammonia on being heated with normal caustic potash solutions[4] The fact that uric acid solutions do not lose ammonia is however by no means inconsistent with the fact that the uric acid is decomposed into something which no longer behaves like uric acid

The crucial qualitative test for uric acid obtained from blood has always been the murexide reaction The remarkable feature about the voluminous literature on the subject of uric acid in blood is the number of positive results reported for uric acid on the basis of this reaction Most of these positive findings represent probably nothing but delusions All know that since uric acid is abundant in the urine, traces of it must be in the blood and all have probably been more or less disposed to accept anything showing a reasonable resemblance to the murexide test as proving the presence of uric acid But the murexide test is made in the same way as is the xanthoprotein reaction for proteins and the red color obtained in the latter is not so very different from the mixture of red and violet obtained in the murexide reaction Moreover, the xanthoprotein reaction is given not only by the various protein materials in the blood but is also given by many phenol compounds, a number of which occur in blood The murexide

[3] *Zeitschr f physiol Chem* , xxxii p 555, 1901

[4] *Ber d deutsch chem Gesellsch* xxxii p 3266, 1899

reaction is therefore by no means reliable when applied to such unknown mixtures as have been obtained from the blood and this combination of circumstances is probably adequate to account for the many remarkable "uric acid" observations recorded in the literature

There is no doubt, however, that by treating greatly concentrated mother liquor obtained from blood with ammoniacal silver nitrate or with copper bisulphite, traces of uric acid can be obtained This is true not only for abnormal blood which is rich in uric acid but holds also for normal human blood, though the various methods heretofore in use have not been sensitive enough to reveal such traces of uric acid Even in the case of gouty blood containing relatively large quantities of uric acid a great deal of blood (75 cc or more)[5] must be taken in order to obtain even a qualitative test For quantitative determinations Brugsch and Schittenhelm use from 150 to 300 grams, *i e*, nearly 10 per cent of all the blood in the body

The new method described in this paper is based on the highly delicate color reaction with our "uric acid reagent," a phosphotungstic acid solution by means of which one part of uric acid in one million parts of water gives a positive test The color obtained from a quarter of a milligram of uric acid is quite adequate for a quantitative determination Normal human blood, as we have discovered, contains about that much uric acid in from 15 to 25 cc so that on the basis of such volumes we are now able to make quantitative determinations of the uric acid in blood

For coagulating the proteins we use five volumes of boiling $\frac{N}{100}$ acetic acid solution (10 cc of normal acetic acid to a liter of water) The blood is drawn into small, wide-mouth bottles previously weighed and containing a small amount (about 0 1 of a gram) of finely powdered potassium oxalate From the subsequent weight of each bottle is obtained the weight of the blood Five times this weight of $\frac{N}{100}$ acetic acid solution is transferred to an ordinary liter flask and heated to boiling The oxalate blood is then poured into this boiling acetic acid solution and the heating is continued until the solution has again begun to boil The mixture while still hot is filtered The coagulated material on

[5] Brugsch and Schittenhelm *Zeitschr f exp Path u Ther* iv p 442, 1907

the filter paper is transferred back into the flask (by means of a small spoon or a spatula), about 200 cc of boiling water[6] are poured over it and it is allowed to stand a few (5) minutes This mixture is then filtered through the same filter as was used for the first filtration The filtrate in the receiving flask should be very nearly as clear as water and will be found to be so if the original blood was promptly shaken with the oxalate so that no clotting has taken place

If clotting has occurred the coagulation and washing of the blood is a little more complicated The clot leads to so much bumping in the boiling acetic acid solution that it is not practicable or safe to try to heat the mixture to boiling The filtration is therefore made earlier The partially coagulated clot is then broken up with a glass rod, transferred to a mortar and there ground into a paste in the presence of hot water This suspension is then poured on the filter The protein material on the filter is then washed as before with about 200 cc of hot water In this case the combined filtrates are, however, never colorless but more or less reddish On being heated to boiling a second small coagulum will be obtained and the filtrate will then be practically as clear as water

The combined filtrate and wash water containing the uric acid and other soluble materials is further acidified by the addition of 5 cc of 50 per cent acetic acid and is evaporated over a free flame in a suitable porcelain dish[7] to a very small volume (about 3 cc) The liquid is then poured into an ordinary small centrifuge tube and the dish washed with two successive portions of 0 1 per cent lithium carbonate solution, using about 2 cc for each rinsing, any solid material adherent to the sides of the evaporating dish being removed by rubbing with a rubber tipped stirring rod To the liquid in the centrifuge tube, which at this stage should not be greater than 10 cc in volume, is added 5 drops of 3 per cent silver

[6] For this washing we use water, not $\frac{1}{100}$ acetic acid solution, because if the latter is used the coagulum will give off more or less of the blood pigment and the filtrates are less clear

[7] Deep (half globular) dishes 10 cm in diameter and having a capacity of about 250 cc are very good for this purpose While free flames are the most convenient for the concentration of the uric acid solutions, care must of course be taken not to char the contents toward the end of the operation Unless one is willing to watch it carefully at this stage it is safer to finish the concentration on the water bath

lactate solution,[8] 2 drops of magnesia mixture and a sufficient amount of strong ammonium hydrate (10–15 drops) to dissolve the silver chloride The tube is centrifuged for two or three minutes, the supernatant liquid poured off and to the residue are added four or five drops of fresh saturated hydrogen sulphide water and one drop of concentrated hydrochloric acid The tube is now placed for a period of five or ten minutes in a beaker of boiling water in order to remove the excess of hydrogen sulphide Since hydrogen sulphide produces a blue color reaction with the phosphotungstic reagent it is of course important to remove every trace of this substance To secure this a drop of 0.5 per cent lead acetate is added to the contents in the centrifuge tube as it is taken out of the hot water Ordinarily very little or no lead sulphide (blackening) is produced, showing that all the hydrogen sulphide is gone as the lead acetate added is enough to give a heavy black precipitate If such a precipitate is obtained it is safest to heat the tube in the water bath for another five minutes, and then add another drop of lead acetate solution When all the hydrogen sulphide is thus removed the contents of the tube are centrifuged for one or two minutes The supernatant liquid is then transferred by decantation as completely as possible to a small beaker and the inside of the tube washed with a stream of water from a wash bottle, care being taken to disturb as little as possible the solid residue at the bottom of the tube The wash water (which should not exceed 5 cc in volume) is then added to the liquid in the beaker and to this acid solution containing the uric acid is then added 2 cc of the uric acid reagent[9] and 10, 15 or 20 cc of saturated sodium carbonate solution depending on whether the color obtained requires a final dilution to 25, 50 or 100 cc

[8] Silver nitrate is undesirable in this connection because traces of liberated nitric acid are not easily excluded and might destroy some of the uric acid Folin and Macallum used silver sulphate to avoid this danger but the sulphate is very insoluble The lactate is therefore the most serviceable silver solution that we have yet found for the precipitation of uric acid Since several reagents are added drop by drop in this work it is very convenient to keep the solutions in so-called dropping bottles

[9] This reagent as stated in previous papers contains 100 grams of sodium tungstate and 80 cc of phosphoric acid (85 per cent) per liter, all boiled together for one or two hours See this *Journal*, xii, p 239, 1912

This direction with reference to the sodium carbonate to be used may seem somewhat vague to the casual reader but is really a simple matter in the actual work The standard solution must already be made and from a rough comparison it is not difficult to determine whether the unknown is to be diluted to 25, 50 or 100 cc in order to give somewhat near the same depth of color as that of the standard

Three volumetric flasks representing 25, 50 and 100 cc, respectively, must be at hand, and the blue unknown solution is transferred to one of them and diluted with water up to the mark The standard solution obtained from 1 mgm of uric acid when treated with 2 cc of uric acid reagent, 20 cc of sodium carbonate and made up to 100 cc should be made just before the addition of the sodium carbonate to the unknown The latter sometimes needs to be filtered before being transferred to the colorimeter cylinders for the final color comparison In all other respects the determination of uric acid in the blood is the same as the corresponding determination in urine[10]

The calculation of the results obtained from the colorimetric readings is not difficult though it must not be forgotten that it is not practicable to take any particular quantity of blood for the work since the usual thing in clinical cases is that the amount of blood available is so small that one takes all that has been collected The following formula gives the amount of uric acid per 100 cc or more accurately per 100 grams of blood

$$\frac{20\,V}{R\,W} = \text{Milligrams of uric acid per 100 grams of blood}$$

In this formula 20 represents the depth in millimeters of the standard solution used for a comparison and R represents the reading in millimeters of the unknown solution V represents the volume (25, 50 or 100 cc) to which the unknown is diluted and W represents the weight of blood taken for the determination

We have made a great many uric acid determinations in human blood and in the blood of a number of different animals by the new method described above Inasmuch as the data so obtained

[10] Folin and Macallum this *Journal*, xiii, p 363, 1912 Blood, like urine, contains phenol compounds which give a blue color with the uric acid reagent Because of the small amount of uric acid and relatively large amount of other materials in blood we have not attempted to determine the uric acid without any preliminary precipitation with silver

possess considerable interest of their own, we prefer to make them the subject of a separate paper Here we wish therefore to add only the determinations of uric acid previously added to blood, showing that by this method it is possible to determine the minute quantities occurring even in normal human blood

Brugsch and Schittenhelm have recorded a few similar experiments[11] by which they tested the merits of the method which they employed in their quantitative work on the uric acid in blood They, however, first dissolved the uric acid in boiling lithium hydroxide and then added from 100 to 200 mgm of uric acid to each 100 grams of blood Results obtained from such experiments have no bearing on the actual analysis of human blood containing at most only 8–10 mgm per 100 cc

In the experiments recorded below the uric acid solution (1 mgm per cc) was prepared by the help of lithium carbonate, as described in a previous paper,[12] and this solution was added in proportions of from 1 to 10 cc to 100 cc of sheep blood which contains no uric acid, *i e*, less than 0 05 mgm per 100 cc

VOLUME OF BLOOD TAKEN FOR ANALYSIS	URIC ACID ADDED	URIC ACID FOUND
cc	*mgm per 100 cc*	*mgm per 100 cc*
50	0	less than 0 05
25	1	0 96
25	1	0 98
25	2	2 00
15	2	2 00
15	4	3 90
25	4	4 00
20	6	5 80
10	8	7 40
15	8	7 50
10	10	9 40
10	10	9 60

[11] *Zeitschr f exp Path u Ther*, iv, p 447, 1907
[12] Folin and Macallum *loc cit*

A NEW COLORIMETRIC METHOD FOR THE DETERMINATION OF EPINEPHRINE[1]

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(Received for publication, November 30, 1912)

In an earlier paper Folin and Denis described the preparation of a phosphotungstic acid solution which proved extraordinarily sensitive as a reagent for uric acid and which also gave a similar (blue) color reaction with polyphenol compounds One such phenol derivative is epinephrine Solutions containing 1 part of epinephrine in 3,000,000 parts of water give an unmistakable reaction with this reagent According to the seemingly careful comparative studies recently published by Borberg[2] none of the usual color reactions for epinephrine are capable of revealing the presence of this substance in dilutions greater than 1 part in 300,000 parts of water Our reaction is therefore approximately ten times as sensitive as the best chemical tests hitherto proposed and in fact approaches in delicacy the remarkable physiological reactions which have heretofore been employed in testing for epinephrine The elaboration of the reaction into a quantitative method for the determination of epinephrine in commercial suprarenal extracts as well as in "home made" extracts of suprarenal glands proved a comparatively easy undertaking

Through the courtesy of Dr Reid Hunt we were saved the laborious and indispensable task of preparing a sample of pure epinephrine From him we obtained some of the ash-free epi-

[1] "The Council on Pharmacy and Chemistry of the American Medical Association has lately adopted the name Epinephrin for the active principle of the suprarenal glands in preference to using a 'protected' name and it is for this reason that we are using the word in this paper " See Abel *Journ of Pharmacol and Exp Therapeutics*, iii, p 328, 1912

[2] *Skand Arch f Physiol* , xxvii p 347, 1912

nephrine purified by Taveau[3] By the help of this product we determined the chromophoric value of epinephrine in terms of another pure and easily obtainable substance (uric acid) which gives the same kind of a blue color with the phosphotungstic acid Epinephrine gives almost exactly three times as much color as an equal weight of uric acid Our determinations gave figures which varied between 2 90 and 2 95 As even our pure epinephrine probably is not as pure as Kahlbaum's uric acid with which we compared it, the ratio 1 3 must be very nearly correct

With this ratio once established it is no longer necessary to have on hand epinephrine solutions of known concentration in order to determine unknown epinephrine solutions With uric acid, the phosphotungstic acid reagent and a high grade colorimeter available, it is now only a matter of a few minutes' work to assay a given solution of epinephrine Using 10 cc volumetric flasks, as little as three or four hundredths of a milligram of epinephrine, or even less, can be determined with a very satisfactory degree of accuracy

To illustrate A freshly opened bottle of "adrenalin" was used as a stock solution and from it one of us (Cannon) made a series of extremely dilute solutions These together with the original solution were submitted to Folin and Denis for analysis The following figures were obtained

Original "a 1 per cent" solution	Found	0 11	per cent
A diluted to contain 25 parts per million	Found	26	parts
B diluted to contain 12 5 parts per million	Found	11	parts
C diluted to contain 2 5 parts per million	Found	1 9	parts

Having proved to the satisfaction of all of us that we were able to assay accurately pure epinephrine solutions, we next undertook to determine whether the crude extract obtained from suprarenal glands could be assayed colorimetrically for their epinephrine content At first sight this seemed very dubious in view of the fact already established by Folin and Denis that blood contains enough uric acid to be estimated quantitatively, and contains besides phenol derivatives which, because of their reactivity with the uric acid reagent, necessitate a preliminary precipitation of the uric acid before it can be determined quantitatively In this

[3] Hygienic Laboratory Bulletin, lv, p 23, 1909

respect muscle, glands and other organs were found to behave in the same way Fortunately, however, the blue color obtained by adding the uric acid reagent to extracts of blood and tissues other than the suprarenals is so very small compared with the color obtained from the latter as to be practically negligible The suprarenals might of course contain some other specific products, besides epinephrine, capable of giving the same blue reaction as the latter Although there is no reason why such should be the case it seemed desirable and indeed necessary to determine the point experimentally by checking up the values obtained colorimetrically by a standard physiological method

Our extracts were made in the following manner The weighed gland is thoroughly rubbed in a mortar with fine sand and tenth-normal hydrochloric acid The mixture is then rinsed into an Erlenmeyer flask with more tenth-normal acid and water, using in all about 15 cc of the acid for each 2 grams of gland and about three times as much water The acid mixture is then heated to boiling In the presence of so much hydrochloric acid there is no coagulation and the epinephrine is thoroughly dissolved To the boiling mixture is then added a little 10 per cent sodium acetate solution (5 cc for each 15 cc of hydrochloric acid present) and the mixture is again heated to boiling The albuminous material comes down promptly under this treatment Instead of filtering and washing at this stage we transfer the whole mixture (except the sand) to a volumetric flask (capacity 100 cc for each 2 grams of gland) and dilute up to the mark with water The solution so obtained or as much of it as is needed can be filtered or centrifuged, giving finally a solution which is nearly or quite as clear as water Ours were obtained by help of the centrifuge

Notwithstanding the highly interesting and convincing character of Elliott's recent epinephrine determinations by the blood pressure method, we are inclined to think that from the standpoint of quantitative analysis his figures are probably not particularly exact, although the physiological reaction involved appears to be suprisingly regular Epinephrine is extracted with considerable difficulty from coagulated material and it is rapidly destroyed by hot Ringer's solution Elliott probably did not always get out all the epinephrine present and he certainly always destroyed some of it by using the more or less alkaline Ringer's solution

The colorimetric determinations in the extracts described above were made at once as follows

the vessel The medulla and upper portion of the spinal cord were next destroyed by a wire passed into the spinal canal through the orbit and brain case Finally the carotid cannula was connected with a mercury manometer The blood pressure now registered at a maintained level of about 40 mm of mercury

The adrenalin (Parke, Davis and Company's) used as a standard was 1 cc of the original (1 1000) solution in 40 cc of distilled water Thus 1 cc of the standard contained 0 025 mgm An injection of 0 1 cc of this solution, *i e*, 0 0025 mgm, would cause a marked rise of blood pressure By repeated injections of the same dose the first record of the pressure change could be duplicated with remarkable exactness Elliott's statement that the largely denervated circulatory system will respond with almost the accuracy of a chemical balance to any submaximal dose of adrenalin we were able to confirm

The extracts were usually diluted 1 10 or 1 25 immediately before being tested Each was placed in a burette connected with the jugular cannula, which was also filled with the extracts At first 0 5 cc of the extract was permitted to run slowly into the vein If this amount gave a satisfactory submaximal rise of pressure, no further record was made If necessary 1 cc was injected

By previous injections of various amounts of the standard solution, the approximate amount required to produce a rise of blood pressure equal to that caused by the extract could be judged With the writing point of the manometer tracing the same zero line as before the estimated amount of the standard solution was now introduced into the vein If the curve rose to the same height as previously the adrenalin content of the injected amounts of standard and extract was regarded as the same, and could be readily calculated If the curve caused by injecting the standard was slightly lower than that caused by injecting the extract, a larger amount of the standard was given And if this in turn resulted in a curve slightly higher than that of the extract, the intermediary value of the standard was taken as corresponding to that of the extract

The results of the determinations made by the physiological process and by the colorimetric method are given in the following table

TABLE II

Epinephrine as determined (A) colorimetrically (B) by its effect on the blood pressure

KIND OF SUPRARENAL GLAND		WEIGHT OF GLAND	TOTAL EPINEPHRINE BY COLORIMETRIC METHOD	EPINEPHRINE BY ELLIOTT S BLOOD PRESSURE METHOD
		grams	*mgm*	*mgm*
Sheep (1)	1	2 25	5 2	
	2	3 07	4 6	5 0
Sheep (2)	1	3 16	6 1	6 2
	2	2 99	5 6	5 6
Sheep (3)	1	2 66	5 2	5 0
	2	2 66	5 0	5 0
Lamb	1	1 17	2 2	2 3
	2	1 02	2 2	2 5
Cattle		11 69	35 0	37 5

It should be stated in connection with the above figures that Cannon, who made the physiological test, did not know the values obtained by the colorimetric test, nor did Folin and Denis know the values obtained by the blood pressure process until all the figures were ready for comparison

ADDENDUM

It is worthy of note that incidental experiments have proved that the method here described is sufficiently delicate, when applied to blood taken from the adrenal veins, to give a stronger color reaction after stimulation of the splanchnic nerves than before Thus to the various physiological evidences that splanchnic stimulation excites secretion of the adrenal glands is added chemical evidence

NEPHELOMETRY IN THE STUDY OF PROTEASES AND NUCLEASES

FIRST PAPER

By PHILIP ADOLPH KOBER

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(Received for publication, December 1, 1912)

INTRODUCTION

The author in studying ferments, required a method which would reveal, quickly and accurately, any change in the activity of the ferment during the course of an investigation The following method, which is based on the use of a nephelometer, introduced into analytical chemistry by Richards,[1] seems to meet those requirements

The following four requisites can be given for a good method of estimating proteases [2]

1 The substance used for a substrate should be a protein

2 The protein should be soluble in the solution of the ferment, in distinction to a reaction between phases

3 Any and all unchanged protein should be completely precipitable by a convenient reagent

4 This undigested protein should be determinable, accurately and quickly

A suitable protein for peptic digestion is edestin,[3] owing to its solubility in weak hydrochloric acid solution Casein[4] is well adapted for tryptic digestion, owing to its solubility in weak alkali

[1] T W Richards *Zeitschr f anorg Chem* , viii, p 269, 1895, Richards and Wells *Amer Chem Journ* , xxxi, p 235, 1904, Richards *ibid* , xxxv, p 510, 1906

[2] The subject of suitable units for proteases will be discussed in the near future

[3] Fuld and Levison *Biochem Zeitschr* , xi, p 473, 1907

[4] Gross *Arch f exp Path* lviii, p 157, 1907

solution, and yeast nucleic acid in dilute solutions is similarly adapted for the study of nucleases

Edestin[5] is easily and quantitatively precipitated as edestan from its acid solution by 12 per cent sodium chloride solution Casein can be completely thrown down by a weak acetic acid solution and other precipitants, while yeast nucleic acid can be rendered wholly insoluble by a slight excess of hydrochloric acid

By using the specially devised nephelometer (easily made from the Duboscq colorimeter) described below, one can determine the amount of any undigested substance which is precipitated in the form of a suspension by these reagents, and thus follow the digestion of edestin with pepsin, of casein with erepsin and trypsin, etc , and yeast nucleic acid with nucleases quantitatively, with an error of less than 2 per cent As a study of precipitants for casein and for yeast nucleic acid is still in progress the results on edestin only will be given in this paper

METHOD

(a) *Description of the instrument and its construction*

As the nephelometer described by Richards yields its best results only on taking a large number of readings (from ten to twenty),[6] and since for ferment work so much time for readings is objectionable, an improvement was highly desirable

A similar instrument of greater accuracy, one that yields reliable results with a few readings comparable to those obtained with a Duboscq colorimeter, would greatly enhance the value of the nephelometer for physiological chemists No doubt the Richards instrument sufficed for the purpose for which Richards designed it and therefore efforts to improve it were heretofore superfluous Since, however, the instrument is not desired now to yield a correction to some other analytical process, but to form the basis of the analytical process itself and to yield all the figures of the determination, greater accuracy is required

[5] T B Osborne *Journ Amer Chem Soc*, xxiv, p 28, 1902

[6] As stated in a private communication to the author, the Richards instrument rarely departs more than 4 per cent from the true value on a single reading, the mean of these readings is within 2 per cent, but to attain accuracy within 0 5 per cent, many readings are needful

Without going into the various considerations connected with the improvement of the nephelometer, it is sufficient to say that the optical workmanship of the Duboscq colorimeter proved to be an excellent basis on which to build a nephelometer, and the following therefore represents an attempt to obtain greater accuracy [7]

The change from colorimeter to nephelometer, and *vice versa*, can be made in a few minutes with the simple, easily made additions described below As most laboratories have a Duboscq colorimeter, an additional useful instrument can be produced in this way with little expense

The essential differences between the new and the older nephelometers are as follows

1 In the new instrument, a plunger changes the height of liquid under observation

2 A shutter supplements the action of the plunger in cutting off the amount of light

3 The new nephelometer eliminates three possible sources of error the meniscus, the indirect reflection of light from suspended matter in the lower part of the tubes, and the reflection of light from one tube to another

4 The dark shade reduces any error due to the reflection of light on the eyepiece

5 The new instrument is more adaptable to daylight

6 Less liquid is needed for a test, 6 cc being the maximum amount needed

7 The instrument is inclined, so as to make observations more convenient, and to prevent air bubbles from forming when the plungers are first introduced into the liquid

The accompanying drawing, for which I am indebted to Mr Sugiura, shows clearly enough the arrangements without much further description

The tubes containing the liquid can be made from soft glass having a bore of 15 2–0 3 mm by anybody having a little skill in glass blowing

The nephelometer shown here was made by the author, without any special tools or skill, from small packing-box boards

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[7] Judging only from the readings with the new nephelometer, greater accuracy was obtained but as both instruments were not compared by the same person under similar circumstances, this statement needs further confirmation

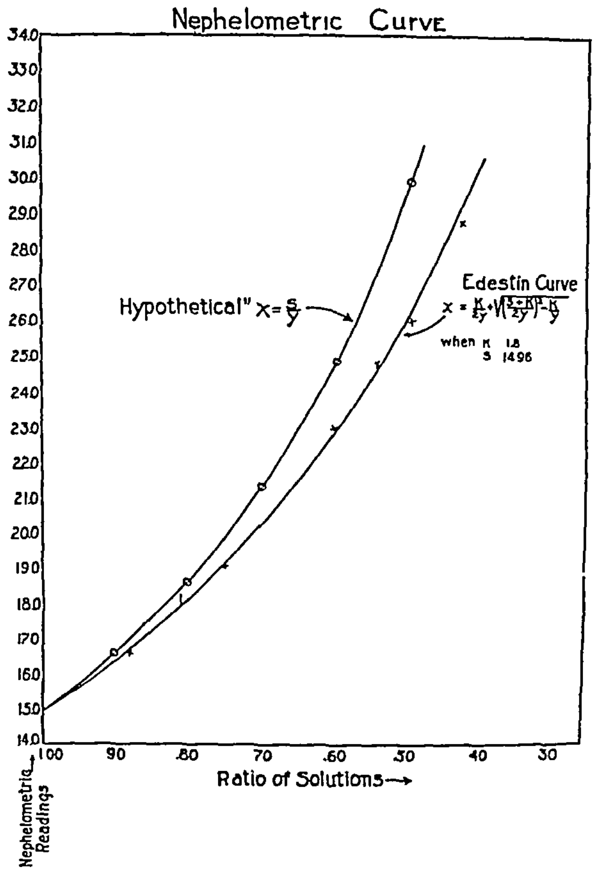

FIGURE 2

figure 2 This curve, as may also be seen, is considerably lower than the "hypothetical" curve where the readings are inversely proportional to the concentration of the solutions Richards and Wells assumed that when solutions of the same height gave the same amount of light, the ratios of the solutions were unity, and that around this ratio the "hypothetical' curve was correct This is shown to be true by the curves in the chart within 1 5 per cent when the ratios are within 10 per cent of unity It was therefore necessary, for Richards and Wells, to adjust the volume of their solutions, until the ratios were within this limit

In the following work with edestin a scheme is presented which practically eliminates this adjustment of volumes

The curve in figure 2 can be expressed in the following equation

$$y = \frac{s}{x} - \frac{(1-x)K}{x^2}$$

where y = height of "unknown" solution, x = ratio of solutions and s = height of standard solution

By s is meant the reading of the nephelometer with the standard solution used as an "unknown," or, in other words, when the ratio of the solutions is unity This serves to eliminate any errors due to faulty light, tubes, plungers, etc

Where K = a constant, obtained by substitution of standardization values

Therefore, if once the value of K is derived for *any given standard solution and height of the standard solution*, the nephelometric readings will give at once, upon calculation, the ratio of the solutions, and thus no further adjustment[9] of volumes is necessary

When the K obtained with one height of standard solution is compared with that of another height, it is found that K is proportional to the height of the standard solution, the equation then becomes for any heights of liquids within moderate limits

$$y = \frac{s}{x} - \frac{s(1-x)k}{x^2}$$

where $k = \frac{K}{s}$

One has, therefore, the choice of using either one or more standard curves and getting the ratios directly from a curve (as in figure 2), without calculation, or taking any suitable standard, adjusting either or both heights of liquids until the amount of light is matched, and obtaining the ratios by substitution in the nephelometric formula given above A more convenient form when expressed in terms of y, is

$$x = \frac{s + sk + \sqrt{(s + sk)^2 - 4sky}}{2y}$$

[9] Of course, it is assumed that solutions which differ from a standard solution very much as may easily be observable macroscopically, will require, as in colorimetric work, a more suitable standard

STANDARD OR CONCENTRATION OF s		UNKNOWN OR CONCENTRATION OF y			MM OF s	MM OF y	THEORETICAL RATIO x	x FOUND IF $x = \frac{s}{y}$	x FOUND IF $x = \frac{s+sk+\sqrt{(s+sk)^2-4sky}}{2y}$
(1-3) NaCl	0 01 per cent edestin	(1-3) NaCl	0 01 per cent edestin						
cc	*cc*	*cc*	*cc*						
10	5	25	5		15 1	26 9	0 500	0 562	0 489
					10 0	18 2	0 500	0 549	0 477
10	5	25	5		14 8	25 9	0 500	0 571	0 504
					9 9	17 6	0 500	0 562	0 493
					8 0	14 0	0 500	0 571	0 495
10	5	25	5		15 1	26 8	0 500	0 563	0 494
					12 0	21 8	0 500	0 550	0 488
					17 0	29 9	0 500	0 568	0 500
5	5	15	5		15 2	27 5	0 500	0 554	0 472
					15 2	26 8	0 500	0 568	0 469
		Average					0 500	0 562	0 491
				Observed y		Corrected y			
25	5	45	5	28 9	13 0	32 3	0 300	0 450	0 275
				11 1	5 0	12 3	0 300	0 451	0 284
25	5	95	5	22 1	10 0	24 6	0 300	0 450	0 282
				28 5	13 0	31 7	0 300	0 456	0 289
95	5	pure H_2O			5 2	28 0			
					4 8	28 0			
		Average					0 300	0 452	0 282

These results are sufficient to show that by the use of either the formula within moderate limits or curves prepared from three or four sets of readings, one can use the nephelometer for the determination of suspended protein and organic substances with the accuracy and with the ease with which the best colorimetric

Since this nephelometric formula seems to hold for a protein of high molecular weight, it was of interest to try its application to argentic chloride suspensions In order to avoid the corrections necessary when very weak solutions are compared, as indicated above with edestin, relatively strong solutions were used

STANDARD s		UNKNOWN y		MM OF s	MM OF y	THEORETICAL x	x FOUND IF $x=\frac{s}{y}$	x FOUND IF $x=\frac{s+sk+\sqrt{(s+sk)^2-4sky}}{2y}$ AND $k=0\ 21$
H_2O	0 00572 per cent AgCl	H_2O	0 00572 per cent AgCl					
cc	*cc*	*cc*	*cc*					
	10		10	15 0	15 0			
					15 1			
					15 0			
					15 0	1 00	1 00	1 00
	10	10	10	15 0	23 8			
					23 8			
					23 6			
					23 5			
					23 7	0 500	0 633	0 500
10	10	30	10	15 0	23 9			
					23 9			
					23 9			
					23 9	0 500	0 628	0 491
	0 001432 per cent AgCl		0 001432 per cent AgCl					
	10		10	15 0	15 1			
					15 0			
					15 0			
					15 0	1 00	1 00	1 00
	10	10	10	15 0	23 6			
					23 4			
					23 5			
					23 5			
					23 5	0 500	0 638	0 509

As may be seen from the results with argentic chloride the nephelometric formula holds for considerable variations in concentrations, the strongest solution being eight times as strong as the weakest, or a ratio of 0 125 It is probable that when corrections are made, especially for the weaker solutions, the result will be still more consistent with the formula It is to be noted that these experiments give $k_{AgCl} = 0\ 21$ and $k_{EDESTIN} = 0\ 12$

(c) *Solutions and reagents*

Edestin solutions were made as follows

1 *Stock solution* 0 1000 gram of Merck's edestin, weighed accurately in a 50 cc beaker, was moistened with sufficient water to make it possible to rub the substance into a paste After thorough rubbing, 3 cc of $\frac{N}{10}$ hydrochloric acid was added which dissolved all but a trace of the suspended substance After allowing the solution to settle for an hour, it was filtered through a well-washed filter paper into a clean, 100 cc graduated flask This was done to prevent foreign particles and fibers of filter-paper from getting into the solutions On bringing the solution up to the mark, adding a few drops of chloroform, shaking and allowing it to stand for twenty-four hours, it was ready for use

2 *Standard solution* 10 cc of this stock solution were diluted to 100 cc, thus making it a 0 01 per cent solution One volume of this standard solution, with one, two or more volumes of 1-3 NaCl solution gave suitable standards for nephelometric work These standards will remain as suspensions one or more hours depending on the dilution In the work given above a fresh standard was made for every determination

3 *Precipitant for acid edestin or edestan* (1-3) NaCl solution

a Stock solution A saturated solution of sodium chloride

b Reagent (1-3) NaCl One volume (250 cc) of saturated NaCl solution, with three volumes of water (750 cc) was found to give satisfactory results in precipitating the edestan completely

(d) *Directions*

For precipitating substances Thus far, satisfactory results have been obtained by adding the precipitant drop by drop from a carefully standardized burette to the standard solutions contained

in a beaker or small Erlenmeyer flask Test tubes should be avoided as it is difficult to shake the solution properly, without adding a stopper or introducing minute air-bubbles into the solution An Erlenmeyer flask or beaker shaken in a rotatory fashion gently is most satisfactory

The tubes may be cleaned and dried with alcohol and ether, or any other suitable way so long as no dust or lint is thereby introduced

For reading the instrument The plunger, being painted with asphaltum paint, obviously cannot be cleaned and dried with alcohol and ether, but may be rinsed with some of the liquid to be examined, or may be wiped carefully with a lint-free cloth or lens paper To use the instrument, it is well to insure proper working conditions by putting into both tubes some standard liquid and comparing the heights If the readings are consistent and practically equal on both sides, one may presume the lighting arrangements and other parts are in proper adjustment

The following suggestions were helpful in obtaining good results

1 The instrument was kept in a separate room, where all the lights could be extinguished at pleasure

2 After the tubes containing the suspensions were in position, the room was darkened completely for a few minutes, to rest the eyes Using the instrument directly after coming from a well-lighted room produced inferior results

3 Both eyes were kept open in reading the instrument This was easily accomplished with the shaded eyepiece, and produced less strain

4 It was found advisable not to make the adjustments too rapidly or too constantly in order to avoid inaccuracies due to eye-strain The final adjustment was made only after relaxing the eyes for a few minutes The eyes were used alternately, the approximate adjustment was made with one eye and the final always with the other

5 When much nephelometric work was done necessitating going to and from a strongly lighted room often, smoked glasses were found restful and advantageous

SUMMARY

I A micro-chemical method for following the digestion of a soluble protein, edestin, based on the use of a nephelometer is given

II A new and sensitive nephelometer easily made from and into a Duboscq colorimeter is described

III The readings of the nephelometer plotted against the ratios of the solutions, for a given standard solution and a given height of standard, seem to follow a uniform curve which can be expressed in the equation $y = \frac{s}{x} - \frac{s(1-x)k}{x^2}$ where y = height of "unknown" solution, s = height of standard solution, x = ratio of solutions

The studies of various precipitants for protein and other organic substances in dilute solutions are in progress with the view of extending the application of this method generally The ease, the rapidity and the accuracy of the method would make it very useful, if the proper precipitants can be found By proper dilution it can be used for large amounts of substances, and is sensitive enough to determine 0 00002 gram with a percentage error of less than 2 per cent The determination of casein in milk and the estimation of minute quantities of ricin are receiving immediate attention

THE PREPARATION AND PROPERTIES OF A COMPOUND PROTEIN, GLOBIN CASEINATE

By T BRAILSFORD ROBERTSON

(*From the Rudolph Spreckels Physiological Laboratory of the University of California*)

(Received for publication, December 2, 1912)

I have elsewhere pointed out[1] that the investigations of Hardy[2] indicate that the proteins in blood sera are united to form a complex which, we may imagine, bears a somewhat similar relation to the individual proteins which constitute it as the proteins bear to the polypeptides out of which they are constructed, and I have furthermore suggested that the biochemical and immunological specificity which is displayed by animal fluids and tissues may possibly be attributable, directly or indirectly, to the existence of these complexes

The only compounds of proteins with one another which have so far been isolated and studied in detail are those which the various protamines form with such proteins as egg albumin, gelatin, hemi-elastin, casein and edestin [3] These compounds are formed on bringing the two proteins together in weakly alkaline (ammoniacal) solution (Kossel) Gay and Robertson have pointed out, however, that it is necessary to have present an excess of the protamine component, otherwise the compound does not readily flocculate [4] Hunter describes clupeine caseinate, which may serve as a type of these compounds, as being insoluble in cold and very slightly soluble in hot water, somewhat soluble in dilute acids and readily precipi-

[1] T Brailsford Robertson *Univ of Calif Publ Physiol* , iv, p 25, 1911, *Die physikalische Chemie der Proteine*, Dresden, 1912, p 126

[2] W B Hardy *Journ of Physiol* , xxxiii, p 251 (Appendix), 1905

[3] A Kossel *Deutsch med Wochenschr* , vii, 1904, cited after A Hunter *Zeitschr f physiol Chem* , liii, p 527, 1907

[4] F P Gay and T Brailsford Robertson *Journ of Exp Med* , xvi, p 479 1912

tated from these solutions by ammonia It is soluble in salt solution On digestion with pepsin-HCl, the casein moiety undergoes hydrolysis, depositing paranuclein, while the protamine moiety remains unhydrolyzed In all of the compounds of clupeine with various proteins which Hunter investigated, the combining weight of the protamine was always considerably smaller than that of the other component of the compound Thus 2 5 parts of casein combine with 1 part of clupeine to form clupeine caseinate, while the compound of clupeine with edestin contains no less than 8 5 parts of edestin by weight to every one of clupeine

Globin is generally considered to be a member of the histone group of proteins,[5] and, according to Kossel,[6] the histones are built up around a large protamine nucleus to which they owe their pronounced basic qualities One might anticipate, therefore, that globin would behave analogously to the protamines and form similar compounds with the less basic proteins

I find that if casein dissolved in dilute alkali be mixed with excess of globin, similarly dissolved in dilute alkali, a coagulum-like precipitate results which would appear, by its behavior, to be a compound of globin with casein, analogous in many respects to the protamine caseinates The method employed in preparing this substance in bulk was as follows

The globin was prepared from ox-blood corpuscles by a method which I have described in detail elsewhere in this number[7] The preparation employed in these experiments (designated Preparation II in the communication cited) is somewhat tinged with haematin and yields somewhat deeply colored, brown solutions (in 2 per cent concentration) The casein was Eimer & Amend's C P Casein "nach Hammarsten," specially purified by a method which has been described elsewhere[8]

In preliminary experiments it was found that, in order to bring about the formation of a precipitate in a globin-casein mixture, it was necessary to avoid the presence of an excess either of casein

[5] Fr N Schulz *Zeitschr f physiol Chem*, xxiv, p 449, 1898

[6] A Kossel and F Kutscher *Zeitschr f physiol Chem*, xxxi, p 165, 1900, A Kossel and H Pringle *ibid*, xlix, p 301, 1906, A Hunter *loc cit*

[7] T Brailsford Robertson this *Journal*, xiii, p 455, 1913

[8] T Brailsford Robertson *Die physikalische Chemie der Proteine*, Dresden, 1912, p 32

or of alkali Rough estimations led to the impression that, given a favorable alkalinity,[9] the proportion of globin to casein should be about 2 to 1 in order to secure a maximum yield of the precipitate

Globin (1 5 grams per 100 cc of solvent) dissolves extremely slowly in $\frac{N}{50}$ or $\frac{N}{50}$ KOH solution, while it readily and completely dissolves in $\frac{N}{10}$ KOH and the concentration of KOH may then be reduced to $\frac{N}{50}$ by the addition of HCl, while stirring, without causing any permanent precipitate to appear[10] On the other hand, if two and a half volumes of a 1 5 per cent solution of globin in $\frac{N}{50}$ KOH be added to one volume of a 2 per cent solution of casein in *0 016* N *KOH* (neutral to phenolphthalein), no precipitate is obtained because the excess of alkali dissolves the globin caseinate, while, if two and a half volumes of a 1 5 per cent solution of globin in $\frac{N}{60}$ KOH be added to a similar solution of casein, a copious precipitate results Accordingly, in preparing the compound it is advisable to dissolve the globin in a slight excess of alkali, and neutralize a portion of this alkali before mixing the solution with the solution of casein

Three hundred and fifty cc of $\frac{N}{10}$ KOH were diluted to 1200 cc and 20 grams of globin were dissolved therein When solution was complete (one to two hours) 150 cc of $\frac{N}{10}$ HCl were added to the mixture while stirring A clear brown solution was thus obtained

Ten grams of casein were dissolved in 500 cc of 0 016 N KOH These two solutions were then mixed, the mixture diluted to 7 liters by the addition of distilled water, thoroughly shaken, and then allowed to settle in tall glass cylinders over night The supernatant fluid was then decanted from the heavy brownish coagulum-like precipitate, which became more granular in the subsequent washings The decanted fluid was very opaque and obviously contained much finely suspended protein material

The precipitate was washed by decantation in 40 liters of distilled water in six successive washings The final washings were perfectly clear It was then washed in 10 liters of absolute alcohol

[9] A slight excess of alkali readily redissolves the precipitate

[10] Analogous phenomena are displayed by casein and other proteins Cf T Brailsford Robertson *Die physikalische Chemie der Proteine*, Dresden 1912, p 70

in two successive washings, collected upon a hardened filter, washed with 2 liters of ether within an incubator over H_2SO_4 and dried in the incubator at 37°C for twenty-four hours It was then pulverized and sifted and returned to the incubator and dried over H_2SO_4 at 37°C for three days The substance was thus obtained in the form of a coarse brownish-gray powder, the yield being 7 5 grams

The phosphorus in two 1-gram portions of this material was determined by von Wendt's modification of Neumann's method[11] with the following results

NUMBER OF SAMPLE	MGM P_2O_5 IN 1 GRAM	PER CENT OF PHOSPHORUS
Sample 1	7 16	0 313
Sample 2	6 86	0 300
Average		0 306

The percentage of phosphorus in casein is 0 887,[12] hence the proportion of casein contained in this preparation of globin caseinate is 34 5 per cent

This preparation of globin caseinate is readily soluble in dilute alkalies, yielding brownish solutions, and in dilute acids, yielding light-colored solutions It is not soluble in distilled water, and the globin caseinate is precipitated from alkaline solutions by neutralization with acid Excess of acid, even of acetic acid, redissolves this precipitate, thus indicating very clearly that the casein is not present as such in a condition of mechanical admixture Globin similarly is precipitated from alkaline solutions by neutralization, but that the properties of the globin have been very materially modified by combination with casein is shown by the *excess* of acetic acid which is necessary in order to redissolve the precipitate which occurs upon neutralization Thus, to 43 cc of an approximately 1 25 per cent solution of globin caseinate in $\frac{N}{10}$ KOH, $\frac{M}{6}$ acetic acid solution was added slowly After adding 19 cc the substance still remained in solution, then a precipitate began to appear, which still persisted until over 65 cc of the acid solution

[11] G von Wendt *Skand Arch f Physiol*, xvii, p 217, 1905

[12] O Hammarsten *A Text-book of Physiological Chemistry*, trans by J A Mandel, 6th Ed, New York, 1911, p 615

had been added, when a very opaque light-colored solution was obtained To 43 cc of a 1 25 per cent solution of globin (Preparation II, cf above) $\frac{M}{6}$ acetic acid was similarly added, a permanent precipitate had already appeared after the addition of 16 cc of the acid solution, and 25 cc sufficed to completely redissolve it I continued adding $\frac{M}{6}$ acetic acid until 65 cc had been added A clear brown solution was obtained

It is clear, therefore, that by the union with casein the basic qualities of globin have been decreased and its acid function enhanced Globin caseinate is held in solution by a smaller excess of alkali than that required to hold the same amount of globin in solution, while a greater excess of acid is required to carry globin caseinate into solution than to carry globin into solution On the other hand, the acid qualities of casein have been diminished and its basic function enhanced by the union with globin, for no excess of dilute ($\frac{M}{1}$ or more dilute) acetic acid solution will carry any casein into solution, a stronger acid, such as a mineral acid, is required It is evident that in these respects the properties of globin caseinate are intermediate between those of globin and those of casein

The solution of globin caseinate in dilute acetic acid does not deposit any precipitate even on standing at 55°C for twenty-four hours It does, however, yield a precipitate on boiling for two or three minutes, and this precipitate does not redissolve on cooling, it is not a coagulum of heat-denatured protein, for it is soluble in dilute alkali It is probably, therefore, casein, and we may conclude that globin caseinate is decomposed into its components by boiling in dilute acid solution

To 70 cc of each of the above solutions of globin caseinate and of globin, respectively, in dilute acetic acid were added 5 cc of a 1 per cent solution of Grubler's pepsin puriss sicc , and the mixtures were placed in an incubator at 37°C After two hours a very slight flocculent precipitate had appeared in the globin solution and a dense granular precipitate in the globin caseinate solution which left the supernatant fluid clear After twenty-four hours, the dense precipitate in the globin caseinate digest had completely redissolved and in each mixture there remained only the slight flocculent precipitate first noted in the globin digest The dense precipitate in the globin caseinate digest cannot have been para-

nuclein resulting from partial hydrolysis of casein, for paranuclein, once precipitated in peptic digests, does not redissolve as digestion proceeds[13] Hence we may conclude that in all probability it was casein, and that the first stage in the action of pepsin upon globin caseinate is to decompose it into its components—globin and casein—which subsequently undergo hydrolysis separately

The globin caseinate digest, after twenty-four hours' digestion, contained no casein either combined or uncombined with globin, as it yielded no precipitate on boiling

The following table of comparative precipitation reactions displays certain respects in which the behavior of globin caseinate resembles or differs from that of casein or of globin The solutions employed were all 2 per cent solutions in $\frac{N}{10}$ KOH A + indicates precipitation (or coagulation) and a 0 indicates no precipitation or coagulation

REAGENT	GLOBIN	GLOBIN CASEINATE	CASEIN
Dilute acetic acid (cold)	0	0	+
Dilute acetic acid (boiling)	0	+	+
Half saturated Am_2SO_4	+	+	+
Four volumes of absolute alcohol	0	0	0
Trichloracetic acid	+	+	+
5 per cent NaCl	0	0	0
Half saturated Na_2SO_4	0	0	0
Saturated NaCl	+	+	0 (+ on heating in the presence of excess of the salt)

I prepared 2 per cent and 1 per cent solutions of globin caseinate in $\frac{N}{10}$ KOH and determined the refractivities of these solutions and of $\frac{N}{10}$ KOH at 16° in a Pulfrich refractometer, reading to within 1′ of the angle of total reflection A sodium flame was the source of light The following were the results obtained The values headed a are calculated from the formula $n - n_1 = a \times c$, where

[13] Lubavin Hoppe-Seyler's *Med-Chem Untersuch*, Berlin, 1866, p 463, A Kossel *Verhandl d Berl physiol Gesellsch*, *Arch f (Anat u) Physiol*, 1891, p 181, T Brailsford Robertson this *Journal*, iii, p 95, 1907

n is the refractive index of the solution, n_1 that of the solvent and c is the percentage of protein in the solution

SOLUTION	n = REFRACTIVE INDEX OF SOLUTION AT 16° C	a
$\frac{N}{10}$ KOH	1 33552	
1 per cent globin caseinate	1 33711	0 00159 ± 0 00008
2 per cent globin caseinate	1 33879	0 00164 ± 0 00004

A duplicate set of determinations yielded identical results

In order to assign to each observation its due weight in determining the average value of a for each of these preparations, it is necessary to add together the observed values of $n - n_1$ for the two solutions and divide this sum by the sum of the concentrations employed (= 3) Proceeding in this way [14] we obtain

$$a = 0\ 00162 \pm 0\ 00005$$

Now the value of a (= change in refractive index of solvent due to 1 per cent of protein) for casein is 0 00152,[15] while the value of a for globin is 0 00169 [16] Hence if the refractivity per gram per 100 cc of solution of globin caseinate were the algebraic sum of the separate refractivities of its components, since, as we have seen, globin caseinate contains 34 5 per cent of casein, the value of a for globin caseinate should be

$$\frac{34\ 5 \times 0\ 00152 + 65\ 5 \times 0\ 00169}{100} = 0\ 00163$$

which is identical, within the experimental error, with the value experimentally ascertained

I have elsewhere shown[17] that the refractivity of the mixed (or combined) proteins of blood sera is equal to the sum of the refractivities of the separate constituent proteins, and, as I have stated, there is much reason to believe that these proteins are bound together to form a compound protein complex We may

[14] T Brailsford Robertson *Die physikalische Chemie der Proteine*, Dresden, 1912, Chapter 13

[15] T Brailsford Robertson *Journ of Physical Chem*, xiii, p 469, 1909

[16] T Brailsford Robertson this *Journal*, xiii, p 462, 1913

[17] T Brailsford Robertson this *Journal*, ix, p 179 1912

therefore conclude that *the refractivity of a compound protein is an additive function of the refractivities of its components*

CONCLUSIONS

1 Globin caseinate may be prepared by mixing two parts by weight of globin with one part by weight of casein, each in faintly alkaline solution

2 Globin caseinate in aqueous solution displays properties intermediate between those of globin and casein, indicating that the acid function of globin is enhanced by union with casein and the basic function of casein by union with globin

3 Globin caseinate is not decomposed by dilute acetic acid in the cold It is decomposed into globin caseinate by the action of boiling dilute acetic acid or by a brief action of pepsin in the presence of dilute acetic acid

4 The change in the refractive index of $\frac{N}{10}$ KOH due to the introduction of 1 per cent of globin caseinate is $0\ 00162 \pm 0\ 00005$

5 The refractivity of a compound protein is an additive function of the refractivities of its components

ON NUCLEASES

THIRD PAPER

By P A LEVENE AND F B LA FORGE

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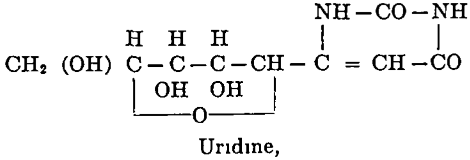
(Received for publication, December 4, 1912)

It was demonstrated through the work of Levene and Jacobs[1] that, compared with purine ribosides, the pyrimidine ribosides possessed a much higher resistance towards the hydrolytic action of mineral acids, and on the basis of this property a method was devised for the isolation of pyrimidine bodies It was later shown by Levene and Medigreceanu[2] that a similar difference existed between the two classes of ribosides in regard to their behavior towards certain enzymes Thus it was found that nucleosidases, which hydrolyzed the purine derivatives, remained without action on the pyrimidine bodies In a previous work by[3] the present writers the fact was brought to light that the difference in the behavior of the two classes of substances towards dilute mineral acids can be removed by the reduction of the pyrimidine base in the pentoside to the corresponding dihydropyrimidine Thus, while uridine was resistant to boiling with dilute aqueous mineral acids, dihydro-uridine behaved under these conditions similarly to inosine, or adenosine, or to any other glucoside

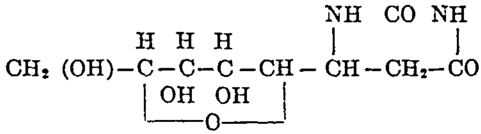


Uridine,
non-hydrolyzable by dilute acids

[1] Levene and Jacobs *Ber d deutsch, chem Gesellsch* xliii, p 3150, 1910

[2] Levene and Medigreceanu this *Journal*, ix, p 65, 1911, ix p 389, 1911

[3] Levene and La Forge *Ber d deutsch chem Gesellsch* , xlv, p 608, 1912



Dihydro-uridine,
hydrolyzable

Thus, dihydro-uridine was shown to possess a chemical structure in every way analogous to other nucleosides, and hence presented a convenient medium for the study of the specificity of nucleosidases. Thus, if it were possible to find tissue extracts which would hydrolyze the nucleosides of one group and failed to decompose the substances of the other group, this find would contain the proof of the existence of specific enzymes capable of acting on nucleosides of only one definite group. In reality it was found that the tissue that possessed the most pronounced action on the purine nucleosides remained without action on dihydro-uridine, and as yet it is impossible to find an enzyme capable of hydrolyzing either the original uridine, or dihydro-uridine. Hence, the mechanism by which the organism brings about the dissolution of pyrimidine nucleosides is as yet undiscovered, but it is made certain that individual nucleosidases possess a limited specific activity.

EXPERIMENTAL PART

In the experiments here recorded the extract of the intestinal mucosa was used as an enzyme solution. It was prepared in the manner described in the papers by Levene and Medigreceanu.

The activity of the extract was tested on adenosine solution and on dihydro-uridine. The presence or absence of hydrolysis was ascertained by the changes in the optical activity of the solution in different intervals after the beginning of the experiment and by the power of the reaction product to reduce Fehling's solution.

The solutions of enzyme and nucleoside were made up to contain one per cent of Henderson's phosphate mixture.

Toluene was used as antiseptic in all experiments.

Experiment 1. (a) About 0.4 gram of dihydro-uridine was dissolved in 7.0 cc. of water, 1.0 cc. of a 10 per cent Henderson's phosphate mixture and 2.0 cc. of the enzyme solution were added.

Original rotation, $[\alpha]_D = -0\ 50°$
After 24 hours, $[\alpha]_D = -0\ 50°$

(b) 4 0 cc of a cold saturated aqueous solution of adenosine were diluted with 0 5 cc of a 10 per cent phosphate mixture and 0 5 cc of the enzyme solution.

Initial rotation, $[\alpha]_D = -0\ 45°$
After 24 hours, $[\alpha]_D = -0\ 20°$

(c) 1 0 cc of Henderson's phosphate solution + 2 cc of enzyme solution and 7 cc of water

Initial rotation, $[\alpha]_D = +0\ 05°$
After 24 hours, $[\alpha]_D = +0\ 05°$

The dihydro-uridine solution gave very slight reduction on prolonged boiling with Fehling's solution An increase in the reducing power at the end of the experiment could not be noticed The adenosine solution showed no reduction at the beginning of the experiment and a marked reduction at the end

The enzyme solution had no reducing power

Experiment 2 (a) To 5 cc of a 25 per cent solution of dihydrouridine was added 0 5 cc of the enzyme solution

Initial rotation, $\alpha_D = -3\ 82\ (\pm 0\ 02)$
After 48 hours, $\alpha_D = -3\ 88\ (\pm 0\ 02)$

(b) Enzyme solution did not possess any appreciable optical activity

(c) About 1 0 gram of adenosine was suspended in 20 0 cc of 1 per cent phosphate mixture and 2 0 cc of enzyme solution, since a part remained insoluble it was not possible to measure the optical rotation of the substance The action of the enzyme solution on adenosine was ascertained by the reducing power of the solution on Fehling's solution

The dihydro-uridine showed no change in reducing power before and after enzyme action

Adenosine solution possessed no reducing power on Fehling's solution at the beginning of the experiment and a very marked reduction after forty-eight hours

The enzyme solution showed no reducing power either at the beginning or at the end of the experiment

THE BIOCHEMISTRY OF THE FEMALE GENITALIA

II THE LIPINS OF THE OVARY AND CORPUS LUTEUM OF THE PREGNANT AND NON-PREGNANT COW [1]

By JACOB ROSENBLOOM

(*From the Laboratory of Biochemistry of the University of Pittsburgh, Pittsburgh, Pa* [2])

(Received for publication, December 4, 1912)

Introduction The biochemistry of the female genitalia is a subject of which practically nothing is known This paper contains the data of a study of the lipins of the ovary and of the corpus luteum of the cow in pregnant and non-pregnant conditions

Methods The desiccation of the material, extraction of the lipins and the chemical analyses were carried out according to the methods described in a former paper[3] with the addition that the cholesterol and cholesterol esters were determined by the excellent method of Windaus

The table on the following page contains the results obtained in this investigation

Conclusions 1 Data are presented showing the percentages of lipins, phospho-lipins, neutral fat, fatty acids and cholesterol in the ovary and corpus luteum of the cow in the non-pregnant state and in the pregnant state

2 The results indicate that there is practically no increase in the above mentioned substances during pregnancy in the cow

[1] A preliminary account of the investigation was published in *Science*, xxxiv, p 222, 1911, *Biochem Bull* , i, p 115, 1911

[2] Most of the analytic data presented in this paper were obtained while working in the Laboratory of Biological Chemistry of Columbia University at the College of Physicians and Surgeons

[3] Hanes and Rosenbloom *Journ of Exp Med* , xiii, p 355, 1911

Table showing lipin composition of ovary and corpus luteum of the pregnant and non-pregnant cow

DRY WEIGHT OF TISSUE	LIPINS		PHOSPHOLIPINS		NEUTRAL FAT		FATTY ACIDS		CHOLESTEROL		CHOLESTEROL ESTERS
			In lipin extract	In dry tissue	In lipin extract	In dry tissue	In lipin extract	In dry tissue	In lipin extract	In dry tissue	
grams	*grams*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
A Non-pregnant ovary, without the corpus luteum											
20 7555	1 426	6 87	57 41	3 94	9 99	0 755	25 9	1 72	6 70	0 146	trace
B Pregnant ovary, without the corpus luteum											
(1)*29 5275	1 838	6 23	55 88	3 48	9 67	0 60	28 10	1 75	6 35	0 40	trace
(2)†20 6400	1 320	6 39	56 87	3 63	9 87	0 63	26 12	1 69	6 84	0 137	trace
C Non-pregnant corpus luteum											
11 201	3 029	27 01	52 14	14 10	10 90	2 95	32 94	8 91	4 02	1 09	trace
D Pregnant corpus luteum											
(1)*10 250	2 820	27 51	52 31	14 90	11 11	3 05	32 62	8 97	3 96	1 09	trace
(2)† 8 364	2 3405	27 95	53 20	14 87	10 69	2 99	32 01	8 95	4 20	1 17	trace
E Ovary with corpus luteum (non-pregnant)—Averages of A and C											
		16 95	54 77	9 02	10 44	1 85	29 42	5 31	5 36	0 77	trace
F Ovary with corpus luteum (pregnant)—Averages of B and D											
	(1)*	16 87	54 09	8 94	10 39	1 82	30 36	5 36	5 15	0 74	trace
	(2)†	17 17	55 03	9 25	10 28	1 81	29 22	5 32	5 52	0 80	trace

Length of foetuses 10–50 cm † Length of foetuses 50–150 cm

THE FATE OF PROLINE IN THE ANIMAL BODY.

By H D DAKIN

(*From the Herter Laboratory, New York*)

(Received for publication, December 9, 1912)

Since nothing appeared to be known as to the fate of proline in the animal body, it seemed desirable to make some experiments in the hope that some light might be thrown upon the changes which this amino-acid may undergo

As a first step in the investigation, observations were made on the behavior of proline when added to blood used for perfusing a surviving dog's liver, using essentially the method which has been so successfully applied by Embden, and secondly, an examination was made of the influence of proline on the glycosuria and acidosis of dogs under the influence of phlorhizin

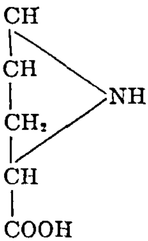
Proline, when added to blood used for perfusing a surviving dog's liver, leads to no increase in the normal formation of aceto-acetic acid, nor is the acetoacetic acid excretion of glycosuric animals markedly increased by the administration of proline It is clear, therefore, that proline is not to be classed with phenylalanine, tyrosine or leucine in having acetoacetic acid for a common catabolic path

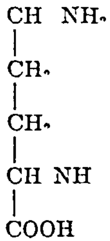
On the other hand, administrations of proline to the glycosuric animal was found to result in a marked increase in sugar output, so that it would appear that proline should be grouped with glutamic and aspartic acids, alanine and glycine, all of which have been shown by Lusk,[1] Ringer and others to be capable of furnishing glucose

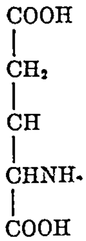
The formation of glucose from proline must necessarily involve disruption of the ring and it is of interest to recall the similarity in structure of the amino-acids, proline, glutamic acid and orni-

[1] *Zeitschr f physiol Chem* , lxvi, p 106, 1910

thine, each of which has five carbon atoms Glutamic acid has been shown by Lusk to be capable of sugar formation in the glycosuric animal and preliminary experiments by the writer indicate that arginine and hence ornithine also possesses this property It would appear, therefore, that these three amino-acids are closely related both structurally and in the changes they may undergo in the animal body


Proline


Ornithine


Glutamic Acid

Liver perfusion experiments

The proline used in the following experiments was obtained from gelatin in the usual way It was purified by repeated solution in alcohol, conversion into the crystalline copper salt and decomposition of the latter with sulphuretted hydrogen, followed by careful recrystallization About two-thirds of the product was the racemic variety and one-third optically active

The experiments on the effect of adding proline to blood used for perfusion of a surviving liver were made in the same way as in the case of experiments previously reported [2]

ADDED SUBSTANCE	WEIGHT OF DOG	VOLUME OF PERFUSION FLUID	TIME OF PERFUSION	ACETOACETIC ACID FORMED
	kgm	*cc*	*minutes*	*mgm*
2 grams proline	9	950	45	49
2 grams proline	11 5	1000	50	44
	11	1000	50	51*

* Average of several experiments

[2] This *Journal*, ix, p 139, 1911

Experiments with diabetic animals

The customary experimental conditions carefully worked out by Lusk, in which a starving dog, rendered as free from glycogen as possible, is made fully diabetic by the administration every eight hours of phlorhizin dissolved in sodium carbonate, were not chosen for the following experiments The reason for this is that a markedly increased acetoacetic acid excretion is apt to follow immediately the administration of the alkaline phlorhizin solution This acetoacetic acid excretion may almost completely disappear after a couple of hours and then reappear on a subsequent injection of the alkaline solution This tends to make the acetoacetic acid output irregular

More suitable experimental conditions seemed to be offered by the method of phlorhizin administration described by Coolen[3] to which my attention was drawn by Professor Lusk It is found that if a single gram of finely powdered phlorhizin suspended in 7–8 cc of olive oil be injected aseptically under the skin of a dog, a glycosuria of remarkable intensity and duration is produced In the starving animal a Dextrose Nitrogen ratio of approximately 3 is quickly established and remains substantially constant for several days

A convenient method of experimenting appears to be as follows A dog is given a gram of phlorhizin in olive oil and starved for forty-eight hours A second gram of phlorhizin is then given and a trial determination of the D N ratio is subsequently made A third gram of phlorhizin is then given on the following day and the urine for the "fore-period" is then collected by catheter, washing the bladder out with warm 2 per cent boric acid solution It is essential that the D N ratio of this urine should not vary greatly from the preliminary trial, as it is useless to proceed with the administration of the amino-acid until a constant ratio has been established The proline in each case was administered by subcutaneous injection of a concentrated aqueous solution

The acetoacetic acid and acetone estimations were made by the customary iodine method Glucose was determined by the gravimetric Fehling method and the results confirmed by polarimetric observation

[3] *Arch de pharmacodynamie*, I, p 267, 1895

Experiment I Weight of dog, 10 kgms

PERIOD	HOURS	TOTAL NITROGEN	GLUCOSE	$\frac{D}{N}$ RATIO	ACETO-ACETIC ACID INCLUDING ACETONE	SUBSTANCE GIVEN
				3 04		1 gram phlorhizin
II	16	6 5	21 20	3 14	0 072	1 gram phlorhizin
III	12	6 94	26 13	3 76	0 042	10 2 gms proline = 1 24 gm N; 1 gm phlorhizin
IV	12	6 44	20 13	3 13	0 103	

Experiment II Weight of dog, 6 kgms

PERIOD	HOURS	TOTAL NITROGEN	GLUCOSE	$\frac{D}{N}$ RATIO	ACETO-ACETIC ACID INCLUDING ACETONE	SUBSTANCE GIVEN
I				2 80		1 gram phlorhizin
II	12	2 73	7 28	2 67	0 302	1 gram phlorhizin
III	16	4 11	14 62	3 55	0 348	10 03 gm proline = 1 22 gm N; 1 gm phlorhizin
IV	12	2 47	6 88	2 79	0 475	
V				2 59		

In the first experiment, adopting a D N ratio of 3 1, it is found that using Lusk's method of calculation, an extra glucose excretion of about 8 grams was observed to follow the administration of 10 2 grams of proline In the second experiment, adopting D N = 2 7, 10 03 grams proline gave "extra glucose" corresponding to about 7 grams Lusk[4] found an excretion of 13 grams of glucose to follow the administration of 20 grams of glutaminic acid, so that it would appear that a molecule of glutamic acid or proline may furnish approximately the same amount of glucose

[4] *Amer Journ of Physiol*, xxii, p 176, 1908

SEASONAL VARIATION IN THE IODINE CONTENT OF THE THYROID GLAND[1]

BY ATHERTON SEIDELL AND FREDERIC FENGER

(From the Hygienic Laboratory, U S Public Health Service, Washington, D C , and the Research Laboratory in Organotherapeutics, Armour and Company, Chicago, Ill)

(Received for publication, December 10, 1912)

The numerous results which have so far been published upon the iodine content of the thyroid gland indicate a very considerable individual variation among animals even of the same species It would therefore be expected that a seasonal variation in the iodine content could not readily be detected by analyzing composite samples of glands from a limited number of animals collected at various seasons of the year In fact experiments along this line made by one of us[2] using thyroids from dogs and from sheep confirmed this conclusion More recent determinations given in a paper by N H Martin[3] have shown that composite samples of thyroids collected throughout the year from sheep slaughtered at Newcastle-on-Tyne, England, varied comparatively little in their content of iodine A striking feature of the results however was the uniformly small size of the glands and the relatively high percentage of iodine

[1] The results described in this paper are the outcome of an effort which was inaugurated by Dr Reid Hunt of the Hygienic Laboratory to fix a standard for thyroid used in medicine This need has been recognized by the Committee of Revision of the Pharmacopoeia and so far a tentative description of this product and a proposed iodine content of 0 2 per cent has been adopted In gathering data for this proposed pharmacopoeial description several manufacturers generously supplied samples and information regarding the preparation of their products Additional points arose and it appeared desirable to obtain further data upon the thyroid, particularly of animals other than sheep Towards this end, therefore, coöperation between Armour and Company and the Hygienic Laboratory was secured and the results presented in the following pages were obtained

[2] Seidell this *Journal*, x, p 95, 1911

[3] *Brit and Colonial Druggist*, lxii, p 99, 1912

The experiments to be described in this paper were undertaken particularly to ascertain the extent of the variation in the iodine content of composite samples of the thyroids of animals used as food by man, rather than with the expectation of finding a seasonal regularity in this variation The need of data of this character is evident since in the production of a thyroid preparation of constant physiological activity a knowledge of the variation in the iodine content of the thyroid glands which constitute the raw material for the manufacture of the drug is essential Among other things, the results here shown demonstrate that a continued restriction of the source of pharmacopoeial desiccated thyroid to the glands of the sheep would make practically impossible the production in the United States of a drug of 0 2 per cent iodine content for all but a short period of the year

The samples which furnish the material for the present paper were prepared from thyroid glands obtained in all cases from animals shipped to the Union Stock Yards in Chicago from practically all parts of the United States except the eastern and southeastern States In this way it can be inferred that local influences have been eliminated to a very large extent and the samples represent the average yield of nearly the whole country The collection of the raw material and the preparation of the samples from it was carried out under the personal supervision of one of the writers in the following manner About 2 5 pounds of healthy normal sized glands from adult sheep, beef and hogs were collected on Tuesdays and Thursdays of each week and stored at freezing temperature At the end of two weeks the combined approximately 10-pound sample of glands from each of the three species of animals was freed from connective and other tissue by carefully trimming the individual glands by hand The material was then finely minced and dried to constant weight at 50°C on agate-ware trays Grease was then removed by extraction with petroleum ether in a Soxhlet apparatus and the sample ground to a fine powder The first eight samples of each series were ground by hand in a Wedgewood mortar to pass a 40-mesh sieve All the other samples were ground in a small ball mill to pass a 100-mesh sieve Of the thoroughly mixed powder composing each sample two portions of about 25 grams each were withdrawn, one to be retained in Chicago for analysis and one sent to the

Hygienic Laboratory in Washington The determinations of the iodine were made by the one of us in Chicago from time to time as the samples were collected and independently by the other in Washington after all of the samples had been received The Hunter[4] method was used for both sets of determinations and the results which were obtained in each case were not communicated to the other party until both sets of determinations had been

TABLE I

Sheep thyroids Showing the percentage of iodine and moisture in dried thyroid glands of the sheep collected at bi-weekly periods during one year

SAMPLE NO AND MID DATE OF ITS BI WEEKLY COLLECTION PERIOD		PER CENT OF IODINE AS DETERMINED BY		PER CENT MOISTURE LOST AT 97 5°	PER CENT IODINE CALCULATED TO DRY BASIS
		Seidell	Fenger		
	1911				
1	September 1	0 133	0 14	3 37	0 138
2	September 16	0 148	0 16	4 29	0 155
3	October 1	0 178	0 22	2 50	0 183
4	October 14	0 181	0 19	2 20	0 185
5	October 28	0 211	0 24	3 53	0 219
6	November 11	0 235	0 26	8 34	0 257
7	November 25	0 219	0 25	7 85	0 238
8	December 10	0 176	0 19	6 98	0 189
9	December 24	0 196	0 20	6 70	0 210
	1912				
10	January 7	0 166	0 17	8 33	0 181
11	January 21	0 112	0 12	9 13	0 123
12	February 4	0 123	0 12	8 53	0 134
13	February 18	0 160	0 14	1 67	0 163
14	March 3	0 044	0 04	7 84	0 048
15	March 17	0 104	0 11	3 30	0 108
16	March 31	0 092	0 10	0 87	0 093
17	April 14	0 062	0 06	8 78	0 068
18	April 28	0 083	0 08	1 05	0 089
19	May 12	0 065	0 07	9 23	0 072
20	May 26	0 038	0 04	8 86	0 042
21	June 9	0 130	0 13	9 58	0 144
22	June 23	0 249	0 24	10 54	0 278
23	July 7	0 294	0 30	12 34	0 335
24	July 21	0 188	0 19	14 75	0 221
25	August 4	0 185	0 19	11 27	0 209
26	August 18	0 235	0 23	10 43	0 262

[4] A Hunter this *Journal*, vii, pp 321–349, 1910

TABLE II

Beef thyroids Showing the percentage of iodine and moisture in dried thyroid glands of the beef collected at bi-weekly periods during one year

SAMPLE NO AND MID DATE OF ITS BI WEEKLY COLLECTION PERIOD	PER CENT OF IODINE AS DETERMINED BY		PER CENT MOISTURE LOST AT 97 5°	PER CENT IODINE CALCULATED TO DRY BASIS
	Seidell	Fenger		
1911				
1 September 1	0 277	0 30	3 16	0 286
2 September 16	0 307	0 31	2 05	0 314
3 October 1	0 240	0 25	0 66	0 242
4 October 14	0 327	0 36	2 34	0 335
5 October 28	0 303	0 35	1 40	0 308
6 November 11	0 332	0 35	8 15	0 361
7 November 25	0 225	0 25	7 24	0 243
8 December 10	0 200	0 22	7 80	0 217
9 December 24	0 123	0 14	6 15	0 131
1912				
10 January 7	0 101	0 11	7 69	0 109
11 January 21	0 045	0 04	9 44	0 050
12 February 4	0 026	0 03	8 41	0 028
13 February 18	0 107	0 10	0 80	0 108
14 March 3	0 053	0 05	7 63	0 058
15 March 17	0 038	0 04	3 88	0 040
16 March 31	0 059	0 06	1 38	0 060
17 April 14	0 068	0 08	9 71	0 075
18 April 28	0 069	0 07	3 14	0 071
19 May 12	0 072	0 08	9 33	0 079
20 May 26	0 095	0 10	10 42	0 106
21 June 9	0 130	0 14	10 58	0 145
22 June 23	0 170	0 19	11 17	0 192
23 July 7	0 288	0 29	13 38	0 332
24 July 21	0 298	0 30	11 12	0 335
25 August 4	0 267	0 27	13 48	0 308
26 August 18	0 310	0 31	10 61	0 347

completed The duplicate series of results are given in tables I, II and III and they furnish very gratifying evidence of the reliability of the Hunter method when applied independently by two analysts to quite a large number of samples It will be noted that variations of more than 0 02 per cent iodine occur only among the first eight samples of each series An explanation of these cases appears to be that these samples as mentioned above were coarsely ground and all the others were very finely ground and certainly most thoroughly mixed

TABLE III

Hog thyroids Showing the percentage of iodine and moisture in dried thyroid glands of the hog collected at bi-weekly periods during one year

SAMPLE NO AND MID DATE OF ITS BI WEEKLY COLLECTION PERIOD		PER CENT OF IODINE AS DETERMINED BY		PER CENT MOISTURE LOST AT 97 5	PER CENT IODINE CALCULATED TO DRY BASIS
		Seidell	Fenger		
	1911				
1	September 1	0 528	0 56	0 55	0 531
2	September 16	0 434	0 46	1 20	0 440
3	October 1	0 416	0 45	0 50	0 418
4	October 14	0 411	0 44	3 83	0 428
5	October 28	0 381	0 41	0 90	0 385
6	November 11	0 273	0 30	11 34	0 308
7	November 25	0 318	0 34	7 74	0 344
8	December 10	0 305	0 33	6 99	0 328
9	December 24	0 337	0 34	7 00	0 362
	1912				
10	January 7	0 275	0 29	7 70	0 298
11	January 21	0 165	0 19	8 40	0 180
12	February 4	0 173	0 18	8 95	0 190
13	February 18	0 219	0 21	2 23	0 224
14	March 3	0 147	0 16	8 15	0 160
15	March 17	0 125	0 12	3 76	0 133
16	March 31	0 188	0 20	1 07	0 190
17	April 14	0 232	0 23	8 46	0 254
18	April 28	0 255	0 26	1 37	0 259
19	May 12	0 244	0 25	8 78	0 268
20	May 26	0 285	0 28	10 82	0 320
21	June 9	0 294	0 31	10 28	0 328
22	June 23	0 344	0 35	11 92	0 391
23	July 7	0 341	0 34	11 49	0 385
24	July 21	0 301	0 31	10 21	0 335
25	August 4	0 398	0 39	13 97	0 463
26	August 18	0 381	0 41	11 01	0 428

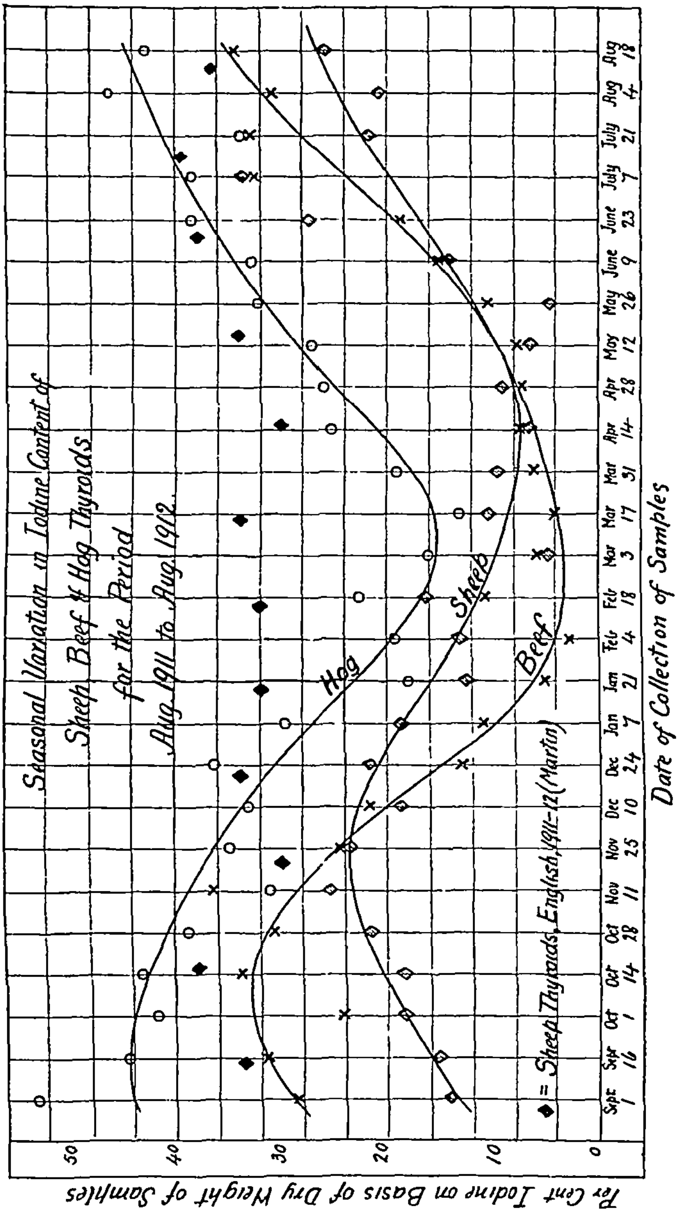
Before discussing the results of the iodine determinations in the samples, the following data upon the weight and size of the glands at the various seasons should be given At the beginning of the experiment there were no particular reasons for suspecting a seasonal variation in the size of the gland, consequently the weights and number of glands in the several lots prepared for the iodine determinations were not taken The averages here shown are therefore based upon figures obtained from time to time by counting out and weighing certain numbers of glands These results are as follows

ANIMAL	AVERAGE WEIGHT IN GRAMS PER SINGLE THYROID GLAND COLLECTED IN	
	Summer and Fall	Winter and Spring
Sheep	5 5	8 5
Beef	25 0	60 0
Hog	9 0	9 0

By comparison of the above results for sheep and beef glands with the diagram showing the seasonal variation in iodine content it will be seen that for the portion of the year during which the iodine content is lowest, namely, December to May, the size of the gland is greatest It therefore follows that this seasonal change in iodine content and size of the glands accords with the frequently made observation that in general an inverse proportion exists between the size of the glands and the iodine content

An examination of the results as recorded in the accompanying tables and diagram shows that, although fluctuations from a regular trend of seasonal change occur, these are small in comparison with the total differences in iodine content between the high and low season Furthermore, the results show that the seasonal variation nearly coincides in the three species of animals studied The average iodine content for the months of June to November is in general about three times that for the months of December to May The maximum and minimum date for each of the three animals can not be selected with certainty, but in so far as can be judged from the diagram the high point for the hog comes first, that is early in September, for the beef it is early in October and for the sheep early in November The fluctuations from the regularity of the curves are no doubt due largely to the individual variations in sex, age, diet, etc , among the animals, the thyroid glands of which form the composite sample in each case

In a study of this kind it is manifestly impossible to follow the change in iodine content which takes place from month to month in the thyroid of an individual animal The best that can be done is to use large numbers of animals and ascertain the average iodine content at a particular period of the year This, however, involves averaging the very wide individual variations which are known to occur Hence to overcome the effect of the individual variations it is necessary to use very large numbers of animals


Seasonal Variation in Iodine Content of
Sheep Beef & Hog Thyroids
for the Period
Aug 1911 to Aug 1912
Hog
Sheep
Beef
◆ = Sheep Thyroids, English, 1911-12 (Martin)
Date of Collection of Samples
Per Cent Iodine on Basis of Dry Weight of Samples
0
10
20
30
40
50
Sept 1
Sept 16
Oct 1
Oct 14
Oct 28
Nov 11
Nov 25
Dec 10
Dec 24
Jan 7
Jan 21
Feb 4
Feb 18
Mar 3
Mar 17
Mar 31
Apr 14
Apr 28
May 12
May 26
June 9
June 23
July 7
July 21
Aug 4
Aug 18

Results obtained in this way can at best show only a general average change which takes place, and the extent of the elimination of fluctuations will depend largely upon the number of glands represented by each composite sample In the present experiment, in which 10 pounds of fresh glands were used for each sample, it appears that the divergence of the several points from the smooth curves, which were drawn as nearly as possible to represent the average of all the points in each series, is about as great in one case as in another Since the total weight of glands was approximately the same for each sample, and the average weight of the individual glands from the three animals varied, the actual number of animals represented by each sample evidently differed considerably, being greatest with the sheep and least with the beef This would therefore indicate that since the averages of a smaller number of beef glands showed no greater fluctuations than the averages of a larger number of sheep glands, the individual variation among sheep glands is probably somewhat greater than among the beef glands, but this point can of course not be definitely concluded

For the sake of comparison the results communicated by Martin[5] are also shown in the diagram and it will be noted that even with these there is a faint indication of a seasonal variation in harmony with the results upon American animals In the case of the English sheep it is seen that the percentage of iodine during the whole year is much above that for the United States sheep even for the portion of the year during which it is highest It may therefore be concluded that the geographical or climatic conditions which are the apparent cause of the high iodine content of the thyroid of English sheep are of sufficient intensity to overcome almost entirely the tendency towards the diminution of iodine content at the particular season of the year at which a lowered iodine content is shown by the present data to occur in the United States

The determination of the moisture and ash in the present series of samples was made as follows One gram of each sample was placed in a weighed porcelain crucible of about 15 cc capacity, and the whole series of samples dried in a water bath at approxi-

[5] *Loc cit*

mately 97 5°C for fifteen hours It was shown by separate experiments that even less than this length of time was sufficient to dry thyroid samples to constant weight The loss in weight having been ascertained and calculated as moisture, the crucibles were placed, in groups of 10 each, in a small muffle furnace previously heated to redness The complete incineration of the organic matter required about one half an hour

In regard to the variations in the percentage of moisture as shown in the tables, it should be mentioned that during the preparation of the samples it became necessary to store quite a few of the sets in covered tin pails after extracting the fat and before powdering This coarse and very porous material evidently reabsorbed moisture while standing in the pails and retained it during the grinding

The individual ash determinations are not reported herewith since it was found that silicious material worn from the inner surface of the ball mill was present in the inorganic residues obtained The proportion of ash varied with the time of grinding necessary to reduce the samples to powder of the requisite fineness Analyses of it indicated the presence of more than 30 per cent of silica The results therefore do not show the true ash content of the several thyroids but only the inorganic content of material prepared under particular conditions In the case of the samples prepared between May 15 and July 15, it was necessary to discontinue the 6-hour day periods of grinding, and run the mill during the night for 15-hour periods These samples are therefore averaged separately and as compared with the averages obtained upon samples ground in a Wedgewood mortar, and in a ball mill for six hours, show strikingly the effect of the longer period of grinding

Percentage of ash

	AVERAGE OF 8 SAMPLES GROUND IN WEDGEWOOD MORTAR	AVERAGE OF 13 SAMPLES GROUND IN PORCELAIN BALL MILL FOR 6 HOURS	AVERAGE OF 5 SAMPLES GROUND IN PORCELAIN BALL MILL FOR 15 HOURS OR LONGER
	per cent	*per cent*	*per cent*
Sheep thyroids	3 11	5 47	12 82
Beef thyroids	3 32	5 86	8 40
Hog thyroids	2 69	4 66	6 58

SUMMARY

It has been shown that a very marked seasonal variation exists in the percentage of iodine present in the healthy normal sized thyroid glands of the sheep, beef and hog There is in general about three times as much iodine present in the glands in the months between June and November as in the months between December and May

A seasonal variation in the size of the fresh glands was observed in the case of the sheep and beef but not with the hog The glands were found to be larger during the months in which the lower iodine content was observed

The independent iodine determinations made upon this series of samples furnish gratifying evidence of the reliability of the Hunter method

The maintenance of a standard of 0 2 per cent iodine in desiccated thyroid prepared for use in medicine will require the use of glands other than those of the sheep and, in addition, the mixing of the product obtained at the high and low seasons of the year

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